INTENSIFICATION OF EFFECTS OF ANTICANCER AGENTS BY USE OF HYPOTHERMIA

by

MASARU TAKAHASHI

From the First Surgical Division, Kyoto University Medical School
(Director : Prof. Dr. CHISATO ARAKI)

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PART I

AUTORADIOGRAPHIC STUDIES OF THE MOUSE TUMOR UNDER THE INFLUENCE OF ANTICANCER AGENTS AND HYPOTHERMIA

INTRODUCTION

The effects of anticancer agents were studied by many investigators through observations on the influence of the drugs upon incorporation of labeled precursors into the nucleic acids in experimental tumors.[8)9)16)40)] They homogenized labeled tumors and counted their radioactivity. This method, however, can not give any information about topographic distribution of radioactivity in the tumor tissue, and it can not be accepted that all cells or cell masses in tumor respond in the same manner to a drug[26)44)]

In the present experiment, effects of anticancer agents on tumors were studied with autoradiogram not only to measure the radioactivity but also to observe topographic distribution in the tumors.

It has been recognized that cell division or tumor growth is arrested at lower temperature[6)19)28)33)]. The process of cell division or tumor growth is thought to be shown in DNA synthesis[21)]. Therefore, an autoradiographic study on DNA synthesis under hypothermia was also carried out.

Experiment (I)

1. Preparation of autoradiogram

Radioactive phosphorus was chosen as the labeled precursor of nucleic acids. The amount of radioactive nucleic acid on autoradiograms is taken as an indicator not only of effects of drugs but also effects of induced hypothermia upon nucleic acid synthesis of tumors.

Total P^{32} Autoradiogram : For the purpose of preventing the escape of radioactive phosphorus from labeled tumors, a rapid procedure was used. The labeled tumor was sliced through its center, fixed in formalin-alcohol (1 : 1) for twelve hours, dehydrated in pure alcohol for one hour and aceton for forty minutes, then embedded in paraffin. The block was placed on a microtome and sectioned into two adjacent slices of different thickness (8 & 4μ), and mounted on glass slides in the usual manner. After deparaffination, the section of 8μ thickness was covered with separation layer through dipping it in 1% celloidin solution. Then it was closely contacted with X-ray film. After the film

had been exposed for two to four days in a desiccator at 4°C, it was developed dy Fuji Rendol and fixed by Fuji Fix. The other section of 4μ was stained with hematoxylin-eosin solution after deparaffination and used for histological comparison with the autoradiogram made from the adjacent section.

DNA P^{32} Autoradiogram[21] : The specimen was fixed in neutral formalin, dehydrated with alcohol and deoxane, embedded in paraffin and two adjacent slices of 8μ in thickness and one of 4μ, which was used for histological study as mentioned above, were obtained and mounted on the glass slides. After deparaffination, one section of 8μ was treated with 10% perchloric acid (PCA) solution for eighteen hours at 4°C, and the other of 8μ was treated as a control with distilled water at 4°C for the same periods. The sections were then washed, dehydrated, covered with 1% celloidin and contacted with X-ray film. In the present experiment, DNA P^{32} autoradiograms were produced by removing RNA with 10% PCA instead of ribonuclease[11)30)34)41)], The removal of RNA with 10% PCA for eighteen hours at 4°C revealed almost equal findings in comparison with those obtained with 0.01% solution of RNA-ase for one hour at 40°C.

2. Relation of the darkreaction to tumor growth

The degree of darkreaction on autoradiograms is thought to be proportional to the amount of beta radiation from P^{32} in the specimen. The mouse of dd strain, inoculated with Ehrlich ascites carcinoma subcutaneously, was given with about 30μC of P^{32}. The section was contacted with X-ray film, and the film was developed after three days of exposure (first autoradiogram). Fourteen days thereafter, the second autoradiogram was made from the section in the same manner as mentioned above.

As the half life of radioactive phosphorus is 14.3 days, the second autoradiogram was reflected with about half as much P^{32} as contained in the original section. As expected, the darkreaction in the second autoradiogram was weaker than that in the first autoradiogram (Fig. 1). The difference of the darkreaction in first and second autoradiogram was offered to the criteria of the darkreaction of autoradiograms in later experiments.

A B

Fig. 1 A : 1st autoradiogram B : 2nd autoradiogram

It is considered that P^{32} turnover rate into a tumor tissue was an indicator of tumor growth, since the more rapidly the tumor grows, the more active the phosphometabolism becomes.

1) Tumors growing at different speeds

Tumors used were two mammary cancers ; MC/4 (35th transfer generation) and MC/5 (28th transfer generation). The tumors grew spontaneously in mice of dd strain and were consecutively transplanted by MIYAWAKI in our laboratory[27]. The growth rate of MC/4 became gradually slower from approximately the 31st transfer generation and

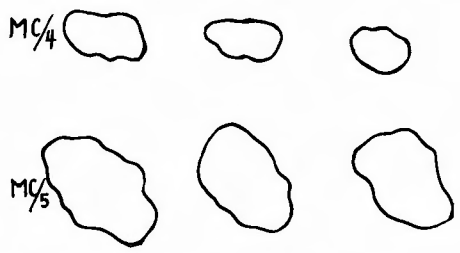
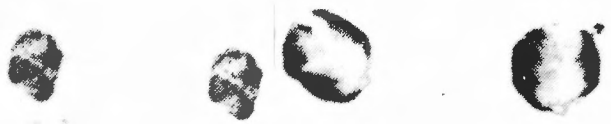


Fig. 2 MC/4 : (35th transfer generation) on 21 days after inoculation
MC/5 : (28th transfer generation) on 15 days after inoculation



A B

Fig. 3 A : MC/4 B : MC/5

the mice carrying it survived longer than those carrying MC/5. Each group of three mice, carrying MC/5 or MC/4 on the fifteenth day or twenty first day after inoculation respectively (Fig. 2), were given with 30μC of P^{32} intraperitoneally and autoradiograms were prepared.

Results :

The darkreactions of rapidly growing MC/5 were more intensive than that of slowly growing MC/4 (Fig. 3). In the autoradiogram of MC/5, there was no darkreaction in the central area, while in the circumferential area a marked intensive reaction was observed.

2) Tumors growing in mice in the environment at 37°C

A different growth response of tumors of mice placed in the environments at two different temperatures (35° and 25°C) was reported by HUNTER[15]. According to his reports, growth of the tumors was more or less suppressed when the mice were kept at 35°C.

A group of mice carrying MC/5 was breeded at 37°C for 24 hours and another group at 25°C. Then they were given with 30μC of P^{32} intraperitoneally and six hours later the tumors were removed to make autoradiograms. It was revealed that the darkreaction of tumors breeded at 37°C was weaker than that at 25°C (Fig. 4).

A B

Fig. 4 A : Tumor of mouse breeded at 25°C B : Tumor of mouse breed at 37°C

3. Response of tumors to anticancer drugs (Mitomycin C & thio-TEPA)

The tumors used were MC/5, Ehrlich carcinoma and NF sarcoma. Male mice of

dd strain, forty to sixty days old, weighing about 20 grams, supplied by animal center in Kyoto University, were used. By our method, rate of transplantability of these tumors were nearly 100%. In most experiments, a group of five tumor bearing mice was tested and another group of five tumor bearing mice was offered for a control group.

The method of transplantation of tumors was as follows ; small pieces (about $1mm^3$) of NF sarcoma from a single donor mouse were implanted aseptically and subcutaneously by troaca, two millions cancer cells of Ehrlich ascites carcinoma were injected subcutaneously and about one cc of homogenate of MC/5 diluted with saline solution was implanted intramuscularly.

The drugs used were Mitomycin C and thio-TEPA. Six days after transplantation of the tumors, these drugs were given intraperitoneally once every day. In case of NF sarcoma bearing mice it was started ten days after the transplantation. The single dose per day was as follows ; Mitomycin C 2mg per kilogram of body weight, thio-TEPA 5mg per kilogram. At the twenty forth hour after the last administration of the drugs which were injected for three or seven consecutive days, 0.1cc of P^{32} solution containing about $30\mu C$ was given to each treated mouse as well as non-treated control animals. They were sacrificed six hours after injection of P^{32} and autoradiograms were prepared.

Results :

1) Response to Mitomycin C
   - Group 1 : MC/5 treated for 3 consecutive days
   - Group 2 : MC/5 treated for 7 consecutive days
   - Group 3 : Ehrlich carcinoma treated for 3 consecutive days
   - Group 4 : Ehrlich carcinoma treated for 7 consecutive days
   - Group 5 : NF sarcoma treated for 3 conscutive days

2) Response to thio-TEPA
   - Group 6 : MC/5 treated for 3 consecutive days
   - Group 7 : MC/5 treated for 7 consecutive days
   - Group 8 : Ehrlich carcinoma treated for 3 consecutive days
   - Group 9 : Ehrlich carcinoma treated for 7 consecutive days
   - Group 10 : NF sarcoma treated for 3 consecutive days

Tumor growth in Group 1 and Group 2 was markedly suppressed in the early stage, that is, for three to five days after beginning the administration of the drugs, but they did not in the later stage in spite of the continuous administration of the drug. The darkreactions in Group 1, as shown in Fig. 5, were distinctly decreased, but in Group 2, no decrease of the reaction was observed when compared with the control. In Group 3, tumor growth was slightly suppressed (Fig. 6), and the darkreactions were remarkably decreased at the sites which corresponded to the histological findings of degeneration of the tumor tissue. But at the other sites, the reactions were almost the same as those of the control (Fig. 7).

There was no significant difference between Group 4 and its control. In regard to the effects of Mitomycin C to tumor growth in Group 5, the agent was effective in two mice of the group. The tumors of the other mice, however, were growing as well as those of the control (Eig. 8). In the tumors, growth of which was suppressed, the darkreac-

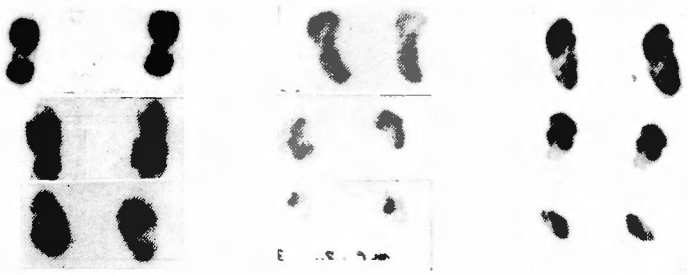


Fig. 5 MC/5 treated by Mitomycin C A : Control group. B : Group treated for 3 days C : Group treated for 7 days

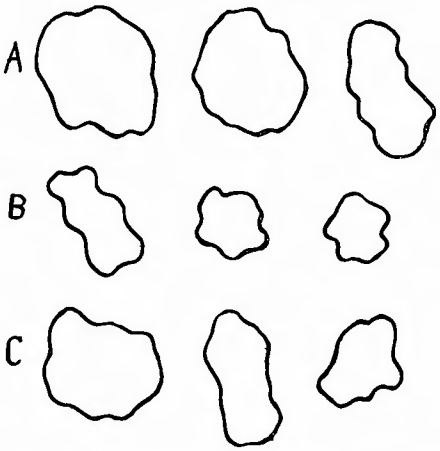


Fig. 6 Ehrlich carcinoma treated for 3 days A : Control group B : Group treated with Mitomycin C C : Group treated with thio-TETA

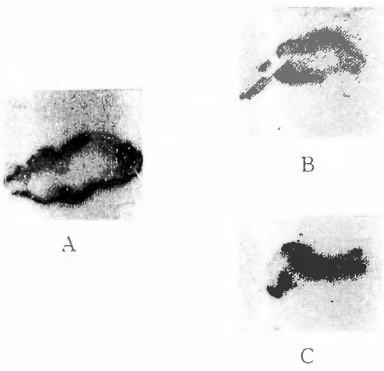


Fig. 7 Ehrlich carcina treated for 3 days A : Control tumor B : Tumor treated with Mitomycin C C Tumor treated with thio-TEPA

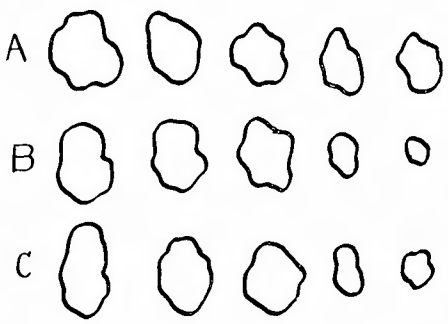


Fig. 8 NF sarcoma treated for 3 days A : Control group B : Group treated with Mitomycin C C : Group treated with thio-TEPA

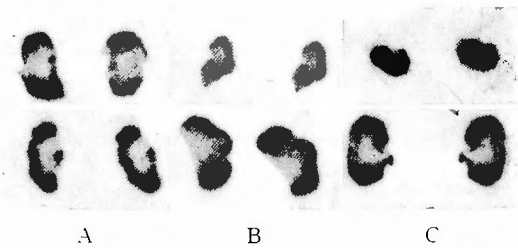


Fig. 9 NF sarcoma treated for 3 days A : Control group B : Group treated with Mitomycin C C : Group treated with thio-TEPA

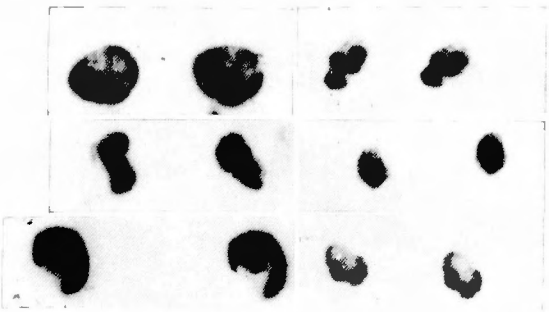


Fig. 10 MC/5 treated with thio-TEPA for 3 days A : Control group B : Treated group

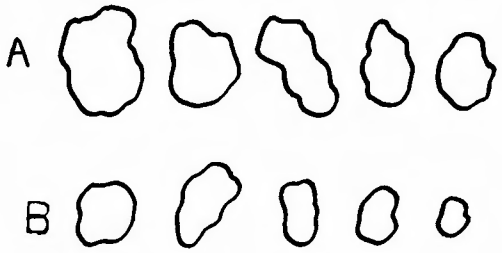


Fig. 11 MC/5 treated with thio-TEPA for 3 days A : Control group. B : Treated group.

tions were shown to be decreased, but not in the other tumors which grew without responding to the agent (Fig. 9).

In Group 6, thio-TEPA appeared to have little effect upon turnover of P^{32} into the tumors (Fig. 10), although it did suppress growth of the tumors (Fig. 11). In Group 7, the same results were observed as in Group 6.

In both Group 8 and 9 the growth of tumors was not suppressed and the darkreactions also were not decreased. In 2 of 5 mice in Group 10 the response of NF sarcoma growth to thio-TEPA was as marked as that in Group 5 (Fig. 8). However, the darkreactions in all of Group 10, on the contrary, appeared to be in the same as in controls, even when tumor growth had been clearly suppressed (Fig. 9).

From these results, it seems that in regard to the response of MC/5, Ehrlich carcinoma and NF sarcoma to Mitomycin C, the decreasing of darkreactions closely corresponds to the inhibition of tumor growth, but as far as thio-TEPA is concerned, there is no parallel relation between the darkreaction and the inhibition of tumor growth (Table).

Table

F.A.	A.T.C. / T. / E.	M C S.T.G.	M C D.P^{32}T.R.	T T S.T.G.	T T D.P^{32}T.R.
3	MC/5	+	+	+	±
	EC	+	±	−	−
	NFS	±	±	±	−
7	MC/5	+	±	+	−
	EC	±	−	−	−

MC : Mitomycin C
TT : Thio-TEPA
S.T.G. . Suppression of tumor growth
D.P^{32}T.R. : Decrease of P^{32} turnover rate
MC/5 : Mammary cancer (Miyawaki)
EC : Ehrlich carcinoma
NFS : NF sarcoma
A.T.C. : Antitumor compound
E. : Effect
T. : Tumor
F. A. : Frequency of administration

Relation between the suppression of growth and decrease of P^{32} turnover rate of tumors treated with anticancer compounds

4. The response of NF sarcoma to Endoxan

It was reported by YAMAGUHI that NF sarcoma was very sensitive to Endoxan, one of the alkylating agent[42].

NF sarcoma bearing mice, 10 days after inoculation, were given with single injection of

20mg per kilogram or 50mg of Endoxan. Since its LD_{50} in mice had not been known yet, the dosage was determined according to YAMAGUCHI's report. Autoradiograms were made in the usual way. DNA P^{32} autoradiograms were also prepared in order to compare the responses of NF sarcoma to Endoxan as above with those of MC 5 to a single injection of Mitomycin C 2mg per kilogram body weight.

Results :

In the total P^{32} autoradiograms the darkreactions of the treated tumors were apparently depressed as compared with those of control and in DNA P^{32} autoradiograms they were also depressed (Fig. 12). On the other hand, in MC/5 treated with the single injection of Mitomycin C, the darkreactions in DNA autoradiograms were slightly depressed (Fig. 13). The data on growth inhibition of NF sarcoma treated by Endoxan will be presented later.

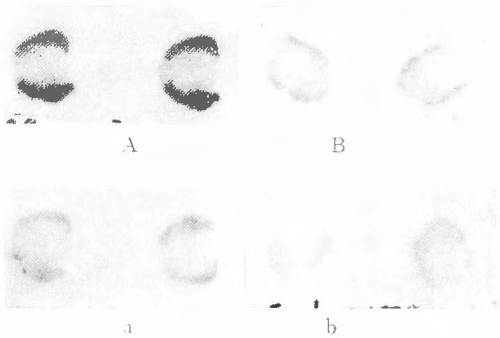


Fig. 12 NF sarcoma given with single injection of Endoxan A Total P^{32} autoradiogram of control tumor a : DNA P^{32} autoradiogram of it B : Total P^{32} autoradiogram of treated tumor b . DNA P^{32} autoradiogram of it

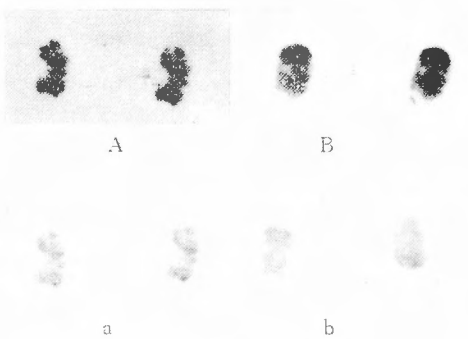


Fig. 13 MC/5 given with single injection of Mitomycin C A : Total P^{32} autoradiogram of control tumor a : DNA P^{32} autoradiogram of it B : Total P^{32} autoradiogram of treated tumor b : DNA P^{32} autoradiogram of it

Experiment (II)

1. Induction of hypothermia in mouse

The normal rectal temperature of mice varied according to the environmental temperature. The average rectal temperature of ten mice was 38.2°C in the environment at 25°C and 36.0°C at 13°C.

Hypothermia was induced by anesthetizing the mice with chlorpromazine (10 mg per kilogram of body weight, intraperitoneally). When the anesthetized mice were left at room temperature, the body temperature gradually decreased to a level 2° to 4° higher than the environmental temperature and remained at this level. When they were placed in an ice box, their body temperature fell rapidly. If body temperature fell below 15°C, some of them would begin to die. By moving the mice out of the ice box or putting them back again into it, the body temperature was kept at about 20°C.

2. P^{32} concentration in blood during hypothermia

It was thought that absorption rate of P^{32} injected into the peritoneal cavity would vary according to the level of body temperature. In order to clarify this point, the radio-

activity of blood, one half, one and two hours after intraperitoneal administration of 30μC of P^{32}, was tested with mice at normal body-temperature and those under hypothermia.

0.5cc of blood from each mouse by cardiac tapping was collected at those times. 0.5cc of blood was diluted with 1.5cc of physiological saline solution containing heparin and 0.01cc of the dilution was then dropped on a round filter paper of 1.5cm diameter and the radioactivity was measured by a G. M. counter.

Thirty minutes after administration of P^{32} its concentration in the blood reached the maximal lebel in both the cooled mice and the mice at normal body-tempeerature. But the counts per minute in the mice at normal body-temperature were at that time three times more than those the cooled mice (Fig. 14).

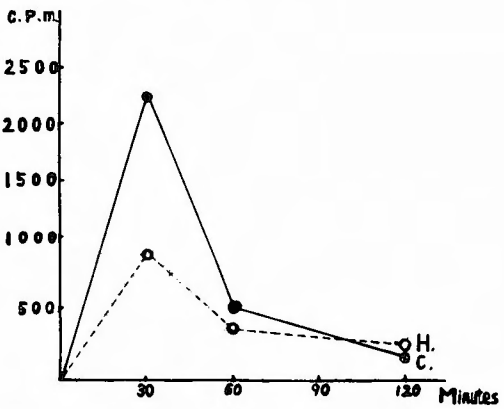


Fig. 14 C. P. M. : Counts per minute H. : Group subjected to hypothermia
C. : Control group

3. Autoradiogram of tumors under deep hypothermia

NF sarcoma and MC/5, were used. Ten days after inoculation the body-temperature of each group of five mice was reduced to 22° to 24°C, and one hour after stabilization of hypothermia 100μC of P^{32} were given intraperitoneally. According to the difference in absorption rate of P^{32}, 30μC of P^{32} were given to the control group at normal body-temperature, in order to obtain the same P^{32} concentration in blood as that of the cooled mice. Then autoradiograms were prepared.

Results :

The uptake of P^{32} after six hours is shown in Figure 15. The intensity of darkreactions in the cooled mice was markedly decreased, although several mitoses were still observed. The darkreactions in the DNA P^{32} autoradiograms and in the total P^{32} autoradiograms appeared to be of the same grade in intensity. Thus under the hypothermia, P^{32} was thought to be incorporated almost exclusively into DNA. In the liver P^{32} uptake was not so depressed as in the tumor (Fig. 16).

4. Autoradiogram of tumors under light hypothermia

NF sarcoma bearing mice, ten days after transplantation were used. Each group consisted of three mice. One group was kept at 32° to 34°C of body temperature, and the other at 20° to 24°C (Fig. 17). P^{32} was given one hour after stabilization of hypothermia. Then autoradiograms of the tumors in each group were prepared.

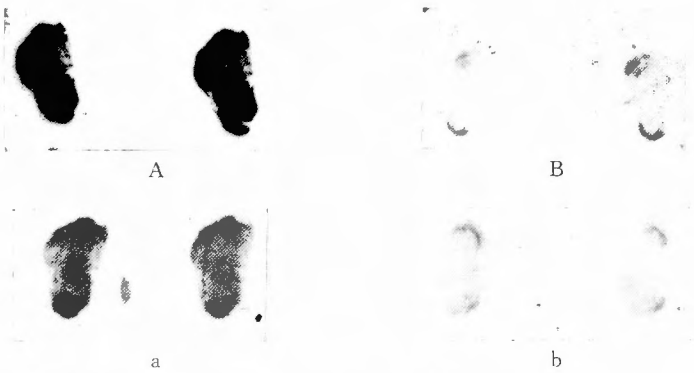


Fig. 15 A : Total P^{32} autoradiogram of tumor of control mouse a : DNA P^{32} autoradiogram of it B : Total P^{32} autoradiogram of tumor of cooled mouse b : DNA P^{32} autoradiogram of it

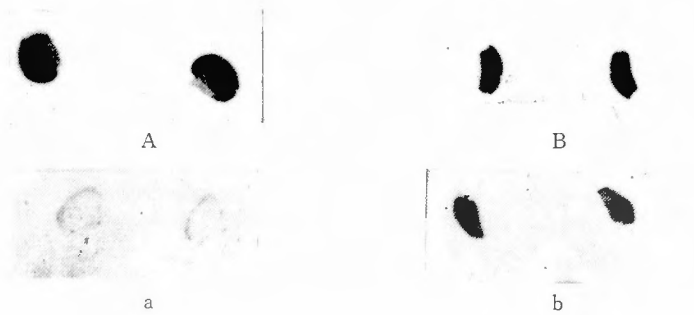


Fig. 16 A : Autoradiogram of tumor of not cooled mouse a : Autoradiogram of tumor of cooled mouse B : Autoradiogram of liver of not cooled mouse b : Autoradiogram of liver of cooled mouse

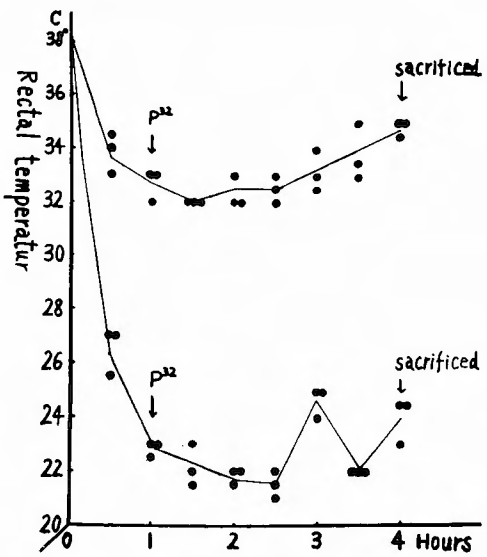


Fig. 17 Rectal temperature of mice during hypothermia

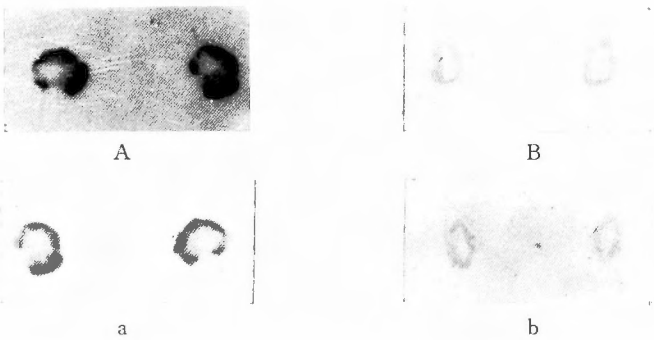


Fig. 18 A : Total P^{32} autoradiogram of tumor during light hypothermia a : DNA P^{32} autoradiogram of it B : Total p^{32} autoradiogram of tumor during deep hypothermia b : DNA P^{32} autoradiogram of it

Results :

It was observed that such light hypothermia did not influence on the darkreaction (Fig. 18).

DISCUSSION

Generally there are two kinds of films to be used in the preparation of autoradiogram ; one is autoradiographic emulsion and the other is X-ray film[18)29)43)]. As autoradiographic emulsion consists of fine particles, its ability of dissolving two points exposed to beta radiation is superior to that of X-ray film. In order to obtain a precise correlation between the darkreaction in autoradiogram and histological findings of adjacent sections, the emulsion should be used. To beta radiation of P^{32}, however, X-ray film is about one to five hundred times more sensitive than emulsion. So X-ray film was thought better to detect a small amount of beta radiation in labeled tumors[18)].

Using the radioactive precursor in experimental tumors, many investigators studied the effects of anticancer drugs[8)9)16)40)]. They measured specific activities of each phosphorus fraction. If tumors were homogenized and then the specific activities per unit weight were measured in each phosphorus fraction, the results would sometimes have no significance. By this method, a rapidly growing tumor in which central necrosis apt to be formed can not be distinguished from slowly growing tumor in which central necrosisis not always to be formed. In the circumferential area of tumors metabolism of cells is most active, while in the central area metabolism is much slower. This was also reported by RIED, et al[25)32)]. In addition, the radioactivity in the tumors treated with anticancer agents is generally decreased. Autoradiograms are thought to be better to detect the decrease of uptake of P^{32} in the tumors which respond to drugs. By this method the locality of distribution of P^{32} can also be shown.

In tumors nucleic acids are synthesized more intensively than in the normal tissues[38)]. The phosphorus metabolism, especially synthesis of DNA, is expected more active in the tumor tissue composed of many dividing cells[4)21)30)]. RNA synthesis, according to DAVIDSON[10)], is more intensive in the growing tissue which is synthesizing protein. The rapidly growing MC/5 showed more intensive radioactivity in its circumferential area than in the central area, where many degenerated cells were found and dividing cells were

quite few. On the other hand, a slowly growing MC/4 has a darkreaction of less intensity and central necrosis was not observed. When the tumor bearing mice were placed in the abnormal environment at 37°C, the darkreaction also decreased corresponding to the suppression of tumor growth. Judging from these two facts, it can be said that the darkreaction in autoradiogram of tumors are probably proportional to their growth rate.

The suppression of growth rate in tumors treated with anticancer drugs, however, was not always proportional to the depression of the darkreaction. The influence of anticancer compounds upon the incorporation of a labeled precursor into nucleic acids was studied experimentally by many investigators. Alkylating agents seemed to have no primary effect upon the turnover rate of P^{32}, although they could suppress growth of the tumors as do the other kinds of anticancer agents[9)40)]. In the present experiments, thio-TEPA, one of the alkylating agents, did not decrease the darkreaction in both MC/5 and NF sarcoma in spite of its suppressive effect on their growth, while Endoxan depressed apparently the turnover rate of P^{32} in NF sarcoma. When Mitomycin C was injected to MC/5 for seven days consecutively, its growth was considerably suppressed for the period of three to five days after the beginning of the injection, and then even with the continued treatment the tumor grew as rapidly as that of control. The darkreaction in the autoradiograms prepared after accomplishment of seven consecutive injections was as intensive as that of control, whereas the autoradiograms prepared after three consecutive injections showed apparently to be decreased. This fact seems to be due to acquired resistance. Cells which are not at a sensitive stage of their division cycle will not be affected by the drug and may survive. The survived cells may acquire some defensive mechanism[24)] and become resistant to damaging effects of a drug administered later, even when they are at their sensitive stage. In some of MC/5 and in Ehrlich carcinoma, when treated with Mitomycin C, the degree of the depression of darkreactions varied in various portions of the tissue. This seems also to be in some relation to the problem of natural resistance. MASIMO found that some cells in a tumor which responded to an agent did not show any morphological changes[26)]. To explain this phenomena, he also suggested natural resistance.

The darkreaction of tumors during hypothermia was depressed conspicuously, even when the concentration of P^{32} in blood of cooled mice were raised to the same level as that in control group. There was no significant difference between the darkreactions in the autoradiograms treated with PCA solution and non-treated ones. During hypothermia, therefore, P^{32} dose not seem to be converted into phosphorus compounds. A slight incorporation of P^{32} into DNA was recognized. It is considered that during hypothermia, nucleic acid synthesis in tumor cells was almost arrested and cell division would consequently be arrested. Many investigators recognized that below the optimal temperature the process of cell division was lost, both in vivo[19)33)] and in vitro[6)28)]. However, in the section of NF sarcoma prepared during the hypothermia in this experiment, a few mitotic figures were observed. In general DNA is thought to be synthesized in advance only for cell division[21)].

As a result of hypothermia (32° to 34°C), the uptake of P^{32} was hardly depressed. Such a light hypothermia, therefore, would not have any marked influence on cell division. SMITH suggested that 32°C was the upper boundary of such a hypothermia which would show the absence of mitosis in human carcinoma by biopsy studies[33)].

The darkreaction in the liver tissue was not so intensively depressed by hypothermia as in the tumor tissues. The bone marrow, one of the most actively dividing tissues in the body, may be influenced by hypothermia as much as that of the tumor tissues.

PART II

INTENSIFICATION OF EFFECTS OF ANTICANCER AGENTS BY USE OF HYPOTHERMIA

Each cell in a tumor is individually in a different phase of its division cycle. It is generally accepted that anticancer agents are the most effective on cells which are in a certain stage of the division cycle[7)14)17)22)39)].

TAKEDA quoted that KARNOFSKY reported the potentiation of anticancer effect by combination therapy of nitrogen mustard and X-ray irradiation on tumor cells based on the assumption that tumor cells, arrested at the premitotic stage by administration of nitrogen mustard, are the most susceptive to X-ray irradiation[39)]. In order to give an effective concentration of anticancer agents at the most sensitive phase of most cancer cells which are dividing at random, SHIRAI proposed a new method of administration of drugs, taking the generation time of cells into consideration. He tried to mentain an effective concentration of drugs in blood through the entire period of the generation time of experimental tumor cells[37)].

If tumor cells in different phases could be artificially synchronized into the same phase, then an anticancer agent could be administered at the most sensitive phase, and probably afford greater therapeutic effect.

NEWTON and WILDY, in 1959, reported that the Hela cells in vitro, subcultured at 37°C, were placed at 4°C for one hour, then replaced at 37°C, and eighteen hours after this as many as 75% of the cells divided within one hour[28)].

The present study was attempted to apply the principle of the in-vitro synchronization method[5)20)36)44)] into the in-vivo experiment in order to intensify the effect of anticancer agents.

1. Phosphorus metabolism after hypothermia

Each group of five NF sarcoma bearing mice, ten days after inoculation, was cooled and their body temperature was maintained at 20°C ± 2°C for four, six and twelve hours respectively, then the body temperature was raised to the level of normal body temperature by warming. Mice of each group were given intraperitoneal injection of 40μC of P^{32} immediately, two hours or five hours after the body temperature returned to the normal level. Then the mice were sacrificed two hours after injection of P^{32}. The autoradiograms were compared with those of the control group.

Results :

1) Four-hours-hypothermia

When P^{32} was injected immediately or two hours after rewarming the darkreactions were almost equal to the control group (Fig. 19).

2) Twelve-hours-hypothermia

When P^{32} was injected immediately after rewarming, the darkreactions were still

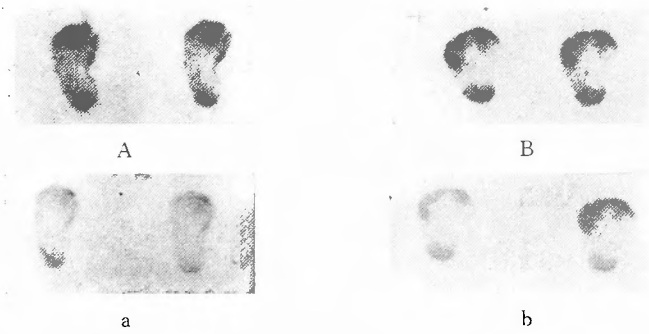


Fig. 19 Autoradiograms of control tumor and tumor subjected to hypothermia for 4 hours. The latter was injected with P^{32} immediately following the release of hypothermia. A : Total P^{32} autoradiogram of control tumor a : DNA P^{32} autoradiogram of it B : Total P^{32} autoradiogram of tumor of cooled mice b : DNA P^{32} autoradiogram of it

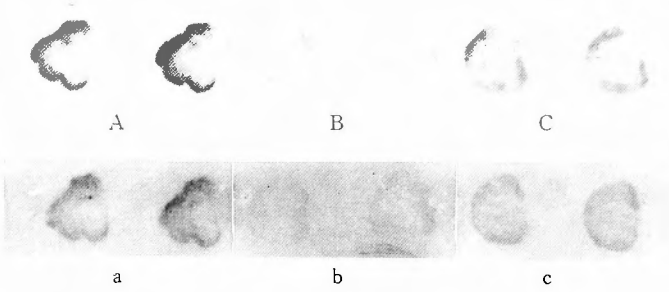


Fig. 20 Autoradiograms of control tumor and tumors subjected to hypothermia for 12 hours A : Total P^{32} autoradiogram of control tumor a : DNA P^{32} autoradiogram of it B : Total P^{32} autoradiogram of tumor immediately after hypothermia b : DNA P^{32} autoradiogram of it C : Total P^{32} autoradiogram of tumor 2 hours after hypothermia C : DNA P^{32} autoradiogram of it

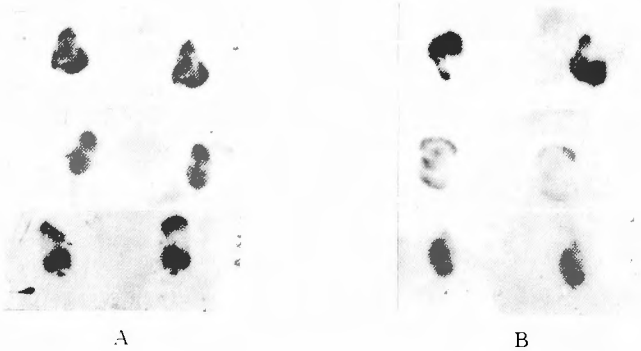


Fig. 21 Autoradiogams of control tumors (A) and tumors subjected to hypothermia for 6 hours (B). The latter was injected with p32 immediately following the release of hypothermia.

markedly depressed comparing with the control group. When P^{32} was given two hours after rewarming the darkreactions recovered considerably but not completely (Fig. 20).

3) Six-hours-hypothermia

When P^{32} was injected immediately after rewarming, the darkreactions were slightly depressed comparing with the control (Fig. 21). When it was given at the second and at the fifth hour after rewarming, they were apparently increased comparing with the control (Fig. 22). The darkreactions in DNA P^{32} autoradiograms were the most intensive when tested at the second hour. At the fifth hour, however, they were found to be decreased (Fig. 23). In autoradiograms of treated groups, the darkreactions seemed to be more intensive in the circumferential area compared with the control group.

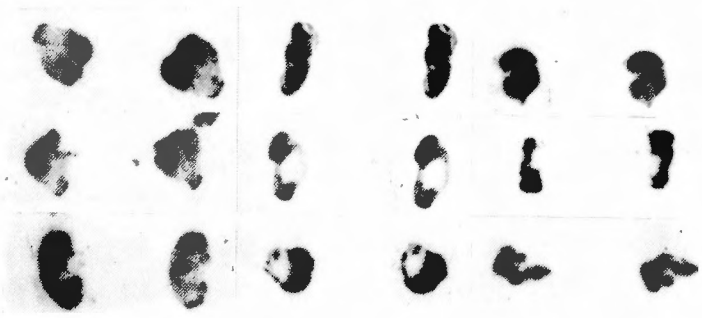


Fig. 22 Autoradiograms of control tumors (A) and tumors subjected to hypothermia for 6 hours. The latter was injected with P^{32} 2 hours (B) or 5 hours (C) after hypothermia respectively.

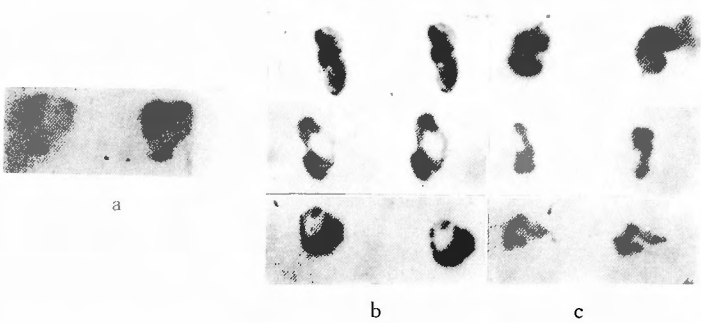


Fig. 23 DNA P^{32} autoradiograms of same tumors as those in Fig. 22 respectively

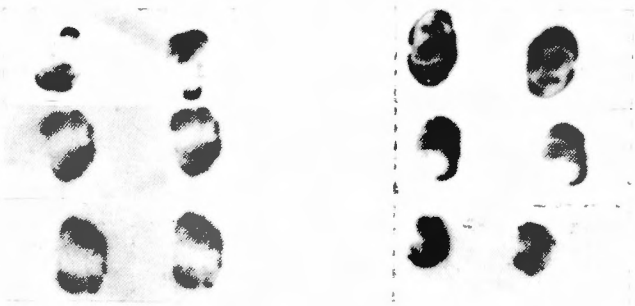
2. Autoradiogram of tumors treated by drugs after hypothermia for six hours

1) Administration of a drug immediately after hypothermia

The synthesis of nucleic acid in the tumors, after release from hypothermia of both six and twelve hours duration, was different from that in the control group. By prolonged hypothermia, irreversible changes may occur in the tumor cells[12)13)]. The purpose of our study did not consist in using hypothermia itself as a direct method of controlling cancer, but as a method of obtaining alteration in metabolism of tumor cells. Therefore, in the following therapeutic experiments hypothermia was applied for six hours.

Three NF sarcoma bearing mice, ten days after inoculation, were kept at a body temperature at 20° ± 2°C for six hours, then the temperature was returned to the normal level. They were injected with Endoxan of 100mg per kilogram of body weight immediately after rewarming. Simultaneously, a control group of three mice breeded at room temperature, was injected with the same drug. Twenty four hours after the injections, all mice were given 30μC of P^{32}. Tumors were removed six hours after the injection of P^{32}, and autoradiograms were made.

The darkreactions of the group injected with Endoxan immediately after release of hypothermia were apparently more intensive than the treated control group without hypothermia (Fig. 24).



A B

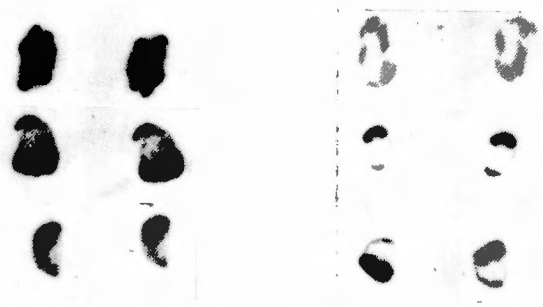
Fig. 24 A : Control group B : Treated group It was injected with Endoxan immediately after hypothermia.

2) Administration of drug two hours after hypothermia

Three NF sarcoma bearing mice, ten days after inoculation, were subjected to hypothermia at 20° ± 2° C for six hours. Then a total of 100mg of Endoxan was administered in three divided doses of 30, 40 and 30mg per kilogram of body weight, consecutively in every one hour, starting two hours after the release of hypothermia. Mice of an equal number were used as a control group and treated in the same way, but without hypothermia. In both groups, twenty four hours after injection, P^{32} was given and autoradiograms were made in the same manner as that of the previous experiment.

Results :

As shown in Fig. 25, the autoradiograms of tumors subjected to hypothermia indica-



A B

Fig. 25 A : Control group B : Treated group It was injected with Endoxan 2 hours after hypothermia.

ted that radioactivity was not observed except in the circumferential areas of the tumors.

3. Experimental therapy after hypothermia

In this experimental therapy of tumors, NF sarcoma was used, because the tumor showed far less variation in regard to its growth rate, as far as our way of transplantation was concerned. The procedure of transplantation was as follows : Female or male mice of dd strain, about forty days old, weighing approximately 20 grams, were used as recipients. Ten days after transplantation, a piece of tissue ($0.1mm^3$) taken from the circumferential area of a tumor, where tumor cells were the most viable, was inplanted subctaneously to the recipient with troaca. In almost cases of the following experiments, the therapy was started on the fifth day after inoculation, since on the forth day the vascularisation was recognized to have been completed in implanted tumors and on the fifth day tumors became large enough to be palpable.

1) Tumor growth in mice treated only with hypothermia for six hours

The body temperature of eight NF sarcoma bearing mice was maintained at $18° \pm 2°C$ for six hours, then returned to the normal level and kept in the same condition as the control group.

Results :

There was no significant difference in growth rate of tumors (Fig. 26, 27) and in survival days (Fig. 28) between the cooled and control group.

2) Administration of anticancer agents after hypothermia

Exper. 1 : Nineteen NF sarcoma bearing mice were separated into three groups. Two groups of six mice were subjected to hypothermia for six hours, then warmed to the normal body temperature. One of these two groups was injected intraperitoneally hourly with 50mg per kilogram of Endoxan for three hours,starting two hours after rewarming (Fig. 29). The other group of six mice was injected with the drug in the same manner starting four hours after rewarming. The third group of seven mice, kept at room temperature as a control group, was treated with the drug in the same manner.

Exper. 2 : Eight mice subjected to hypothermia in the same way as Exper. 1 were injected with a total of 100mg of Endoxan, in three divided doses of 30, 40 and 30mg each

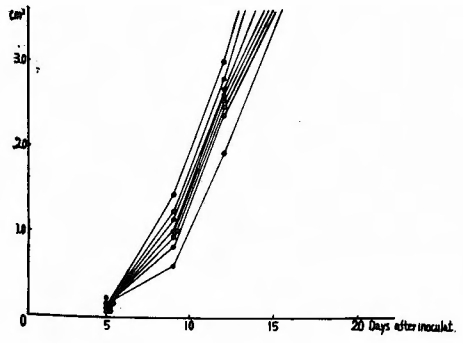


Fig. 26 Growth curve of NF sarcoma without treatment

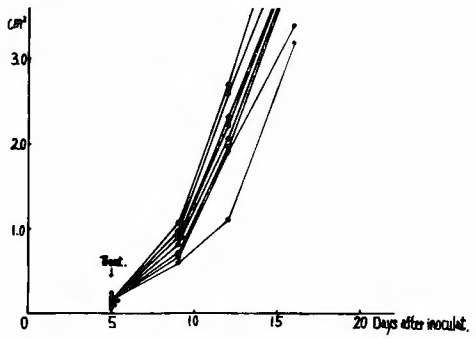


Fig. 27 Growth curve of NF sarcoma treated with hypothermia only Any anticancer drug was not given.

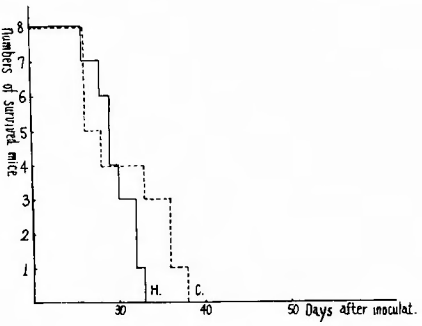


Fig. 28 C : Gontrol group of mice
H : Group of mice sudjected to hypothermia

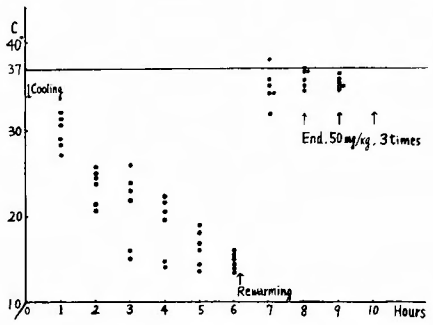


Fig. 29 Rectal temperature of mice during and after the hypothermia

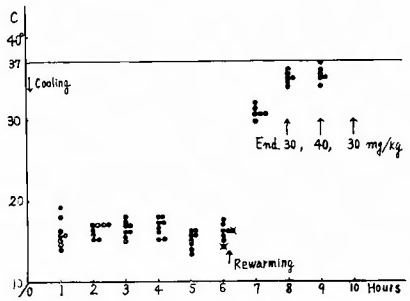


Fig. 30 Rectal temperature of mice during and after hypothermia

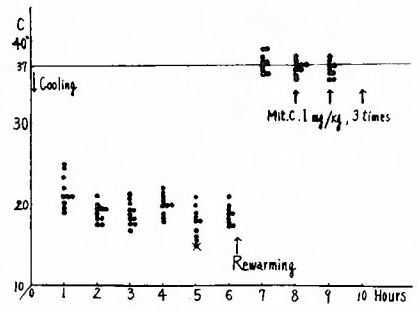


Fig. 31 Rectal temperature of mice during and after hypothermia

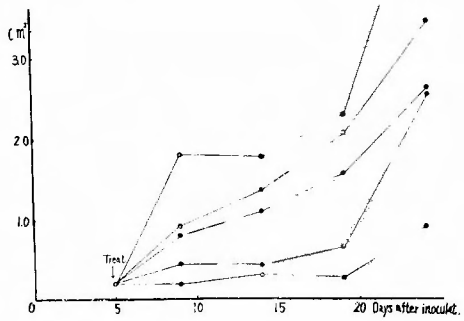


Fig. 32 Growth curve of NF sarcoma treated with Endoxan (150mg/kg) only

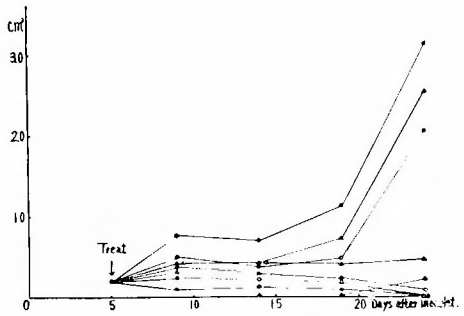


Fig. 33 Growth curve of NF sarcoma treated with Endoxan (150mg/kg) 2 and 4 hours after hypothermia

per kilogram body weight, every hour from two hours after rewarming as shown in Fig. 30. Another eight mice without hypothermia were treated by the same method. The third group of five mice was injected with the same total dose of Endoxan daily for three days without hypothermia.

Exper. 3 : Two groups of nine mice were subjected to hypothermia for six hours. One group was injected with a single dose of 100mg per kilogram of Endoxan one hour after beginning of hypothermia. The other group was injected with the agent in three

divided doses given hourly from two hours after rewarming as in Exper. 2.

Exper. 4 : Ten Bashford carcinoma bearing mice subjected to hypothermia for six hours, were injected with a total of 3mg of Mitomycin C in three divided doses given hourly from two hours after rewarming as is shown in Fig. 31. Another ten mice were given Mitomycin C in the same manner, without being subjected to hypothermia.

Exper. 5 : Eight mice on the third day after inoculation of NF sarcoma were divided into two groups. The tumor was not yet palpable. One group subjected to hypothermia for two hours was injected with Endoxan in three divided doses, starting two hours after rewarming as in the above experiment. The other group was given with Endoxan by the same method without hypothermia.

Results :

Exper. 1 : Although the rate of transplantability of NF sarcoma was usually about 100% and its growth rate was homogeneous, two of seven mice in the control group, one of six mice of the group subjected to hypothermia and treated two hours after rewarming and two of six mice of the group treated four hours after rewarming, had scarcely palpable tumors on the fifth day after inoculation ; the reason is not yet known. In these mice, tumors never started to grow after the treatment. In the remaining five mice of the control, all tumors grew gradually showing some variation on the growth curve (Fig. 32). Of course, their growth was considerably depressed compared with a group which was not treated at all. In the remaining five mice treated from two hours after rewarming, growth of the tumors was suppressed more intensively than that of the control group (Fig. 33) and three of the five tumors soon regressed and disappeared in about three weeks after treatment. At that time, however, the other two tumors, which were growing slowly at an earlier period, began to grow as rapidly as those of the control. In the remaining four mice treated from four hours after rewarming, one mouse died during hypothermia and the tumors of three mice were growing more slowly than those of the control groups. The survival days of most mice in this experiment were almost the same. But four of six mice treated two hours after rewarming, two of five mice treated

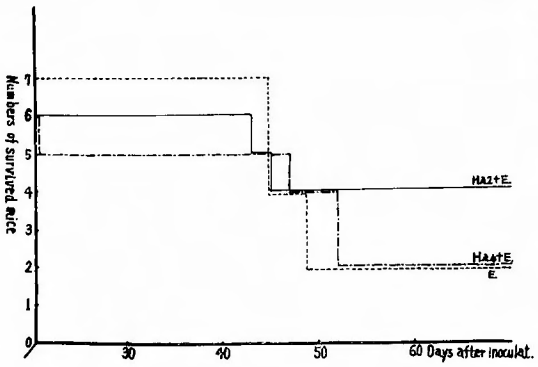


Fig. 34 E : Group of mice treated with Endoxan only H.a.2+E : Group treated with Endoxan 2 hours after hypothermia H.a.4+E : Group treated with Endoxan 4 hours after hypothermia

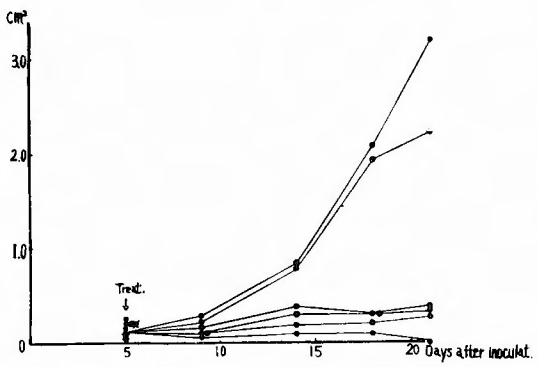


Fig. 35 Growth curve of NF sarcoma treated with Endoxan (100mg/kg) 2 hours after hypothermia

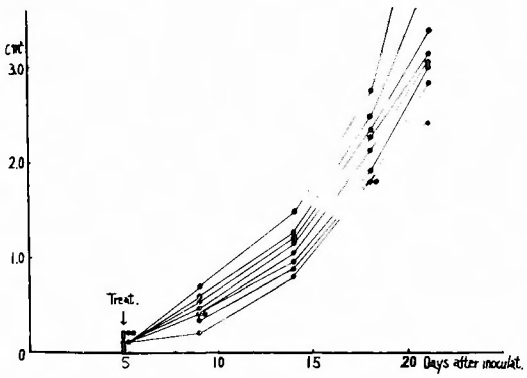


Fig. 36 Growth curve of NF Sarcoma treated with Endoxan (100mg/kg) only, the 3 divided doses of which was injected hourly

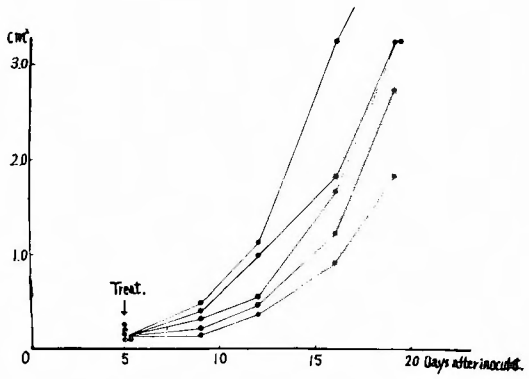


Fig. 37 Growth curve of NF sarcoma treated with Endoxan (100mg/kg) only, the 3 divided doses of which was injected daily

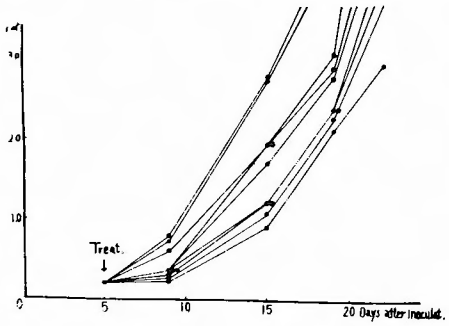


Fig. 38 Growth curve of NF sarcoma treated with Endoxan during hypothermia

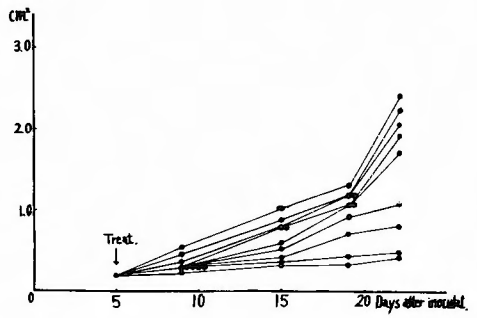


Fig. 39 Growth curve of NF sarcoma treated with Endoxan after hypothermia

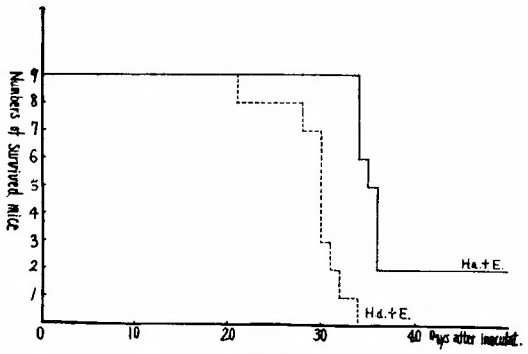


Fig. 40 H.a.+E. : Group of mice treated with Endoxan after hypothermia
H.a.+E. : Group of mice treated with Endoxan during hypothermia

four hours after rewarming and two of seven mice in the control survived for more than three month (Fig. 34).

Exper. 2 : Growth of the tumors subjected to hypothermia, all of which were well palpable on the fifth day after inoculation, were also markedly suppressed for about two

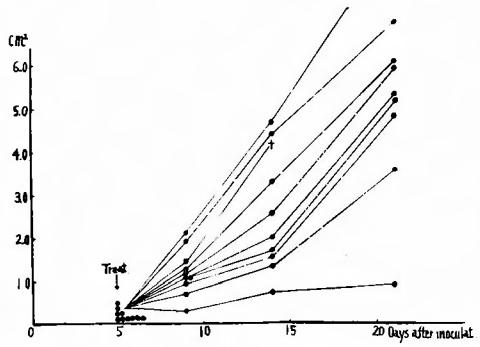


Fig. 41 Growth curve of Bashford carcinoma treated with Mitomycin C only

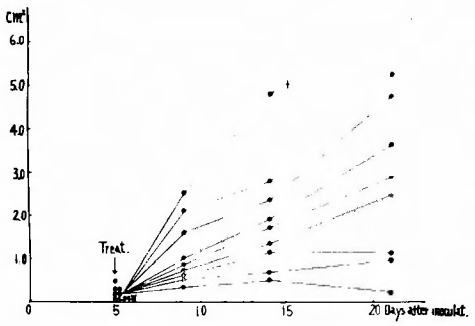


Fig. 42 Growth curve of Bashford carcinoma treated with Mitomycin C after hypothermia

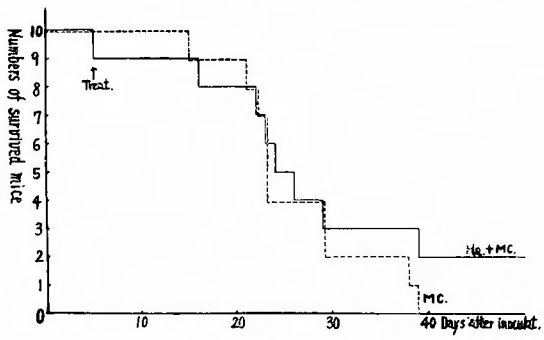


Fig. 43 H.a.+MC : Group of mice treated with Mitomycin C after hypothermia
MC : Group of mice treated with Mitomycin C only

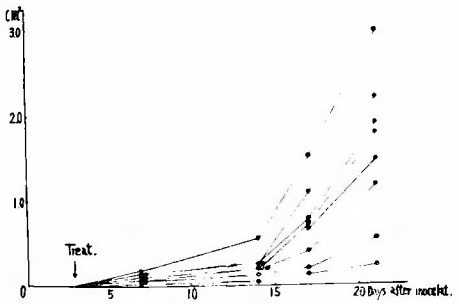


Fig. 44 Growth curve of NF sarcoma treated with Endoxan only

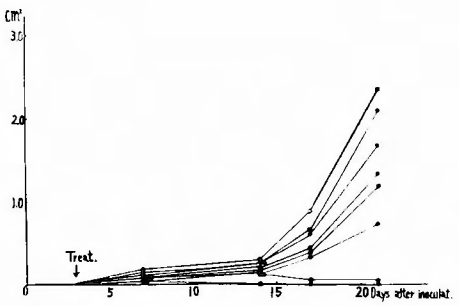


Fig. 45 Growth curve of NF Sarcoma treated with Endoxan after hypothermia for 2 hours

weeks after therapeutic procedure compared with the tumors treated without hypothermia (Fig. 35, 36). There was no significant difference in the survival curves between the group subjected to hypothermia prior to Endoxan administration and the group treated only with Endoxan every hour, although in the latter group two mice died during hypo-

thermia and only one of the remaining mice survived for more than three months after treatment. The tumors of the mice which were injected with the drug every hour were growing no more slowly than those which were treated with the same total amount of the drug administered daily (Fig. 37).

Exper. 3 : In the group injected with Endoxan during hypothermia, the rate of tumor growth was faster and the survival days were shorter than that of the group treated from two hours after hypothermia (Fig. 38, 39, 40).

Exper. 4 : In this experiment growth of the tumors of the group subjected to hypothermia was suppressed compared with that of the control tumors treated without hypothermia (Fig. 41, 42). Two mice of the group subjected to hypothermia have shown no reccurrence of the tumor after three months. One mouse of this group died during hypothermia, and one died after hypothermia. A mouse of the group treated without hypothermia was lost by accident (Fig. 43).

Exper. 5 : There was no significant difference in growth rate of the tumors between the group subjected to hypothermia and the group treated without hypothermia. But in a relatively short period of time after the treatment, the size of tumors of the group treated with hypothermia seemed to be more slightly suppressed than that of the group treated in the same manner without hypothermia (Fig. 44. 45).

DISCUSSION

There are many reports concerning the influence of hypothermia upon tumor growth. Hypothermia was first utlilized as a method of treating human cancers by SMITH and FAY, in 1938[12)33)]. They reported that, with hypothermia and local refrigeration for various periods, from several days to several weeks, the size of tumors was decreased and pains were relieved clinically. On the other hand, ARIER suggested that reducing the body temperature of rabbits for short periods (18°C for six hours, 20°C for eight hours and 30°C for twenty four hours) had no definite, lasting suppressing effect upon growth of tumors[1)]. ARKELL, BISCHOFF, LYMAN and PATTERSON recognized similar results in tumors subjected to hypothermia[2)3)19)31)]. Our data also indicated that hypothermia itself did not suppress growth of NF sarcoma.

As shown in the reports of LYMAN and PATTERSON, stating that tumor growth was arrested under hypothermia in experimental animals, it is assumed that cell division is arrested without any irreversible changes under hypothermia at about 20°C for a relatively short period.

In the autoradiograms of tumor tissue of the cooled mice in which P^{32} was injected two hours and five hours after release from six-hour-hypothermia, the darkreactions obtained after two hours of P^{32} uptake in tumors appeared more intensive than those of the control group. This seems that the metabolism of tumors two and five hours after hypothermia is more active than that of control group.

Moreover, in the DNA P^{32} autoradiograms of the cooled mice in which P^{32} was injected two hours after release from six-hours-hypothermia, the darkreactions, especially in the circumferential area of tumors, appeared more intensive than those of cooled mice in which P^{32} was given five hours after the hypothermia and those of the control group.

A considerable amount of DNA is synthesized during this period in the tumor of the cooled mice, especially in the circumferential area of tumor. In the studies of breast cancer in C3H mice, MENDELSOHN recognized that there was a time lapse of one to four hours between completion of DNA synthesis and onset of mitosis[23]. NEWTON suggested that synthesis of DNA occurs during interphase and it dose not seem to continue at the same rate through the whole period of life span of cells. He reported that DNA synthesis is most active during two phase ; one at early period of interphase, and the other in the end of interphase. According to Swan, DNA synthesis takes place in late interphase[35], and CAMPBELL suggested that DNA is synthesized rather discontinuously, generally at some time during interphase[5].

Our coworkers observed that in Ehrlich carcinoma and Sarcoma 180 bearing mice subjected to hypothermia at about 20°C for six hours, mitotic counts decreased rapidly during the hypothermia and several hours after the animals were rewarmed to normal body temperature, they increased to a higher level than normal. CHÉVREMONT also reported that in tissue culture of chick emblyo, the cells subcultured at 37°C were cooled at 20° to 16°C for twenty four hours and when they were rewarmed, mitotic counts increased markedly in three hours[6].

These reports and our data that DNA synthesis of NF sarcoma became maximum two hours after release from six-hours-hypothermia might indicate that many tumor cells would soon enter into mitosis a few hours after DNA synthesis.

On the other hand, according to DUSTIN, GILMAN, ISHIDATE, MARSHAK and TAKEDA, radiomimetic anticancer agents are the most effective on the cells which are in their premitotic stage of division cycle[7)14)17)22)39]. Thus, Endoxan were administered to NF sarcoma bearing mice, for three hours starting two hours after release from six-hours-hypothermia, since this period of administering the drug was thought to correspond to the premitotic stage of most tumor cells. The results of Bashford carcinoma for Mitomycin C, however, were not so good as those of NF sarcoma for Endoxan. This might be due to inadequate administration of Mitomycin C. It is not elucidated yet at what stage of division cycle of cancer cells Mitomycin C may be most effeective. When Endoxan was administered during hypothermia or immediately after hypothermia, the effects were definitly inferior to those obtained by our method of treatment (chemotherapy after hypothermia), judging from the darkreactions as well as from the therapeutic experiments. In the former groups, at the time of administering the drug, cell metabolism was depressed and most cells might not be so sensitive to the drug. Hypothermia lasting only two hours could not intensify the effects of the drug. This might be due to the fact that under such a light hypothermia metabolism of nucleic acid after release from the hypothermia was not hardly influenced.

SUMMARY AND CONCLUSION

With autoradiogram, the effects of anticancer agents and induced hypothermia on phosphometabolism of various transplantable mouse tumors were studied. Taking the results of this experiment into consideration, a new method of intensifying the effects of anticancer agents was proposed. Tumor bearing mice were subjected to six-hour-hypothermia at

20°C and after release from the hypothermia an anticancer drug was administered at the period when most cancer cells were thought to be in the premitotic stage of division cycle. The results were as follows ;

1) The darkreaction in autoradiograms of the tumors appeared to be proportional to their growth rates.

2) In regard to the effects of Mitomycin C, as far as the tumors used in this study were concerned, decreasing of the darkreaction corresponded to the degree of suppression of tumor growth, but in regard to thio-TEPA, an alkylating agint, it depressed the tumor growth, but not the darkreaction. On the contrary, Endoxan, another alkylating agent, depressed the growth of NF sarcoma as well as the intensity of darkreaction.

3) During hypothermia P^{32} activity of tumors of mice was markedly depressed, but only a slight reaction in DNA P^{32} autoradiogram was still observed. The darkreaction of the liver tissue was not so depressed as that of the tumor tissues.

4) By light hypothermia, P^{32} activity in tumor was not so depressed.

5) When NF sarcoma bearing mice subjected to six-hour-hypothermia at about 20°C were returned to the normal body temperature, the darkreactions in DNA P^{32} autoradiograms were maximum in the period between two to four hours after rewarming.

6) The hypothermia itself did not suppress the growth of NF sarcoma.

7) Endoxan administered in three divided doses hourly starting two hours after release from hypothermia suppressed the growth of NF sarcoma and prolonged the survival days of the mice as compared with Endoxan given alone or given during hypothermia.

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和 文 抄 録

低体温法を利用せる制癌剤の効果増強法

京都大学医学部外科第1講座（指導：荒木千里教授）
高 橋 勝

種々の mouse 移植癌について，腫瘍増殖抑制効果と，腫瘍への P^{32} turnover rate 及び腫瘍内での P^{32} の活性分布の変動との関係に及ぼす2,3の制癌剤の影響を Autoradiogram により検索した．更に担癌マウスに低体温を施行し，低体温時及び低体温解除後に投与された P^{32} の腫瘍組織への ternover 特に DNA への転入について検索した．

1) Mitomycin C 投与は，腫瘍発育の抑制と Autoadiogram の黒化度減少との間に，略々平行関係が認められたが，thio-TEPA では腫瘍の発育を抑制しても，黒化度の減少は認められなかつた．この様にアルキル化剤では腫瘍の発育は抑制されるにも拘らず，燐代謝は低下しないといわれているが，アルキル化剤の一つである Endoxan は NF 肉腫に於て黒化度を著明に減少した．

2) 20°C 前後の低体温時，腫瘍組織に於ては P^{32} turnover rate は著明に低下し，DNA への転入が僅かに認められるだけであつたが，肝組織では腫瘍組織程，黒化度の減少は認められなかつた．又32°C前後の light hypothermia では，腫瘍組織に於ける黒化度は殆ど低下していなかつた．

3) NF 肉腫を移植した mouse に 20°C 前後6時間の低体温を行ない，正常体温に復温した後に P^{32} のとり込みの変動を検索したところ，P^{32} の DNA への転入は復温後2時間目より4時間目で対照以上に強くなつた．

一般に制癌剤は個々の腫瘍細胞についていえば，細胞の分裂周期のうち，ある段階に特に鋭敏に作用するといわれているので，此の鋭敏な時期を撰んで薬剤の投与を行なえば治療効果を増強する事が可能と思われる．一方 in vitro で腫瘍細胞の培養温度を変化させる事により，大部分の細胞の分裂を同期させ得るという事実が判明している．上記の本実験成績をみても，in vivo で，担癌動物を一定の低体温条件のもとにおいた後，正常体温にもどすと，その後或る時期に P^{32} の腫瘍組織特にその周辺部に於ける腫瘍組織の DNA への転入が亢進している事が認められた．そこで担癌動物に一定の低体温法を施行して後復温し，制癌剤の投与を腫瘍細胞の最も敏感な時期を狙つて重点的に行なうという新しい投与方法を試み，次の如き結果を得た．

4) NF 肉腫を移植した mouse に6時間の低体温を行ない，正常体温に復温した後，2時間より1時間毎に Endoxan 3回投与を行なつたものは，低体温を行なわれずに薬剤の投与を行なつた対照群に比べて腫瘍発育抑制効果が優つていた．

5) 低体温6時間のみでは，腫瘍の発育は殆ど抑制されなかつた．又低体温施行中に Endoxan 投与を行なつた場合も制癌剤の効果は増強されなかつた．