The Cytological Effect of Chemicals on Tumors, XIX. Effect of 1-thia-3-azazulan-2-one on HeLa Cells and Two Animal Tumors[1)]

By

Jindo Kobayashi[2)]

Zoological Institute, Hokkaido University

(*With 20 Text-figures and 3 Tables*)

Current interest in cancer chemotherapy has been directed to the action of chemical compounds in special regard to their chemical structures in relation to cellular metabolism (Biesele 1958, 1961). Recent studies have pointed out that there is a significant difference in the level of ribonucleic acid metabolism and its requirement between non-malignant and malignant cells (Takeyama *et al.* 1958, Miura *et al.* 1958). Some metabolic studies have been made with a seven-carbon-ring compound, 1-thia-3-azazulan-2-one[3)] by Miura *et al.* (1958), who reported that the compound inhibited RNA metabolism of rat liver. Ormsbee *et al.* (1947, 1948) reported that a given agent might have certain effects in tissue culture, though not observed in animals.

The present study deals with differential action of TAAO on the proliferation of tumor cells *in vitro* and *in vivo*, with particular attention to cellular metabolism.

The author is deeply grateful to Professor Sajiro Makino for his direction and for going through the manuscript. Gratitude must also be extended to Dr. Yoshiaki Miura, University of Tokyo, who kindly supplied TAAO for this study.

Material and methods: The following experiments were carried out with tumor cells grown in tissue culture. Healthy HeLa cells which proliferated on the bottom of stock culture bottle were treated with 0.2 per cent trypsin in Rinaldini's solution for disjoining of cells. After having been washed with Earle's solution, the cells were planted on 12 × 32 mm cover slips in culture tubes. To each tube was added one milliliter of a supernatant medium of the following formula: 0.5 per cent lactalbumin hydrolysate (Difco) plus 0.1 per cent yeast extract (Difco) dissolved in Earle's solution and inactivated bovine serum in proportion of 85: 15. Each milliliter of medium contained 25 units of penicillin G. The tubes were then incubated at 37°C.

---

1) Contribution No. 558 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

Aided by a grant from the Damon Runyon Memorial Fund for Cancer Research to S. Makino (DRG–563B).

2) Present address: Department of Medical Radiology, School of Medicine, Tokushima University, Tokushima, Japan.

3) TAAO is hereafter used for the abbreviation of 1–thia–3–azazulan–2–one.

After incubation for four to five days, the cells were found proliferated on the cover slip as monolayer (Abercrombie 1961). TAAO was tested for its action at 500, 1000 and 2000γ per milliliter concentration. In all cases, treatment lasted for 24 hours. The cultures were fixed after the treatment with methanol or Carnoy's fixative and stained with May-Grünwald-Giemsa (MGG) (Jacobson and Webb 1952). Toluidine blue at pH 4.6 in phosphate buffer solution was used to identify RNA according to Lison (1953). RNA was selectively colored, and its specificity to RNA was insured by impairment of stainability after treatment with 0.001 per cent RNase. The colorations were blue in both the cytoplasm and nucleolus, while violet in both the nucleus and chromosomes.

Phase microscopy study was made for detailed observations of affected cells; cultures removed from the culture tube were mounted on specially designed slides with a small amount of culture medium and kept at 37°C in a thermobox equipped with a phase contrast microscope during the course of observation.

Rats of Wistar strain weighing 60 to 80 grams, and EM mice weighing 20 to 25 grams, were used for transmission of tumors. The ascites tumors used were the MTK-sarcoma III of the rat and EM-tumor of the mouse which malignantly propagated in each peritoneal cavity. On the 4th day after inoculation of the tumor, the tumor-bearing animals received intraperitoneal injections of TAAO at the following dosage levels: 5000γ, 10000γ and 50000γ per kg for rats and 1000γ, 2000γ and 5000γ per kg for mice. The tumor cells were sampled from treated animals at appropriate intervals after application of the agent. The preparations for cytological observations were made by squashing the cells with acetic dahlia. The materials stained with MGG or toluidine blue were used for the study of RNA in tumor cells.

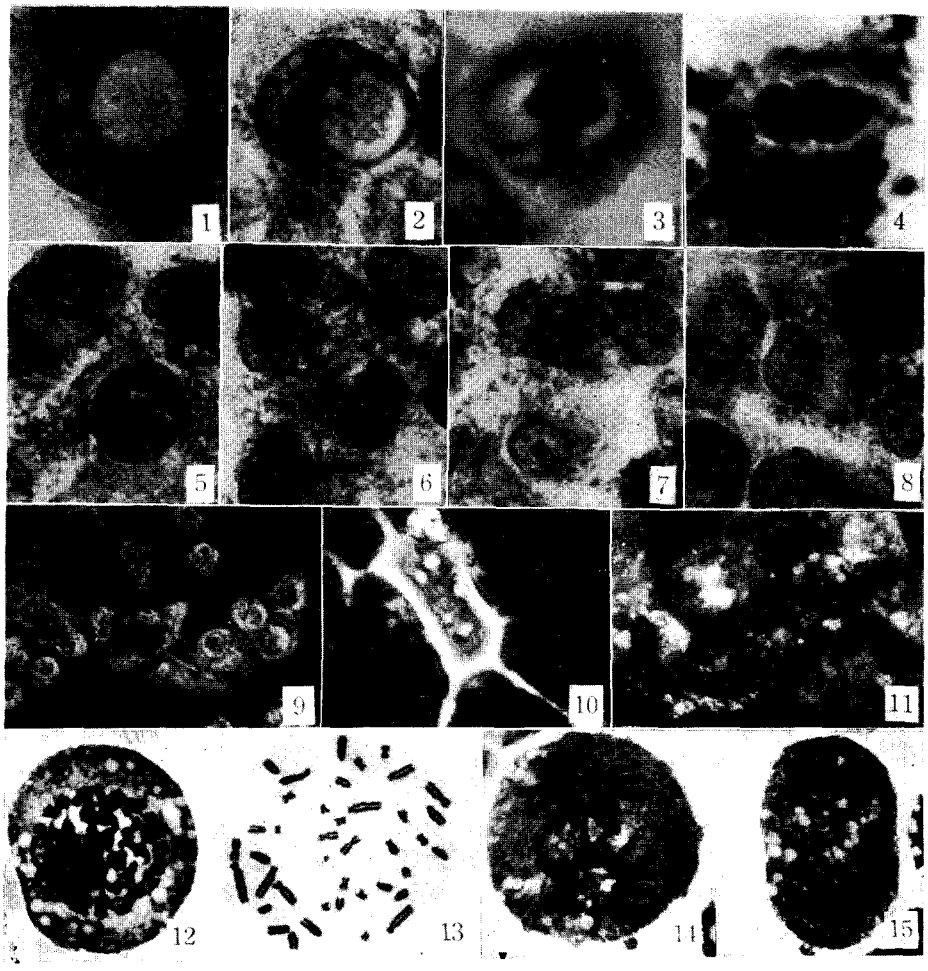
Results

I. *Effects on HeLa cells*

The process of cell-damage at a dosage of 500γ per ml is illustrated in Figures 1 to 8 based on toluidine blue preparations. Treatment with TAAO at 2000γ per ml showed that with 24 hours after treatment almost all cells became spherical in outline, being relatively small in size (Fig. 9); the process of cellular change was rapid, and therefore material was unfavorable for cytological observations. The following descriptions are based on observations of culture cells which were treated at 500γ per ml.

Mitotic frequencies observed in 500γ specimens are shown in Figure 16. Damage to tumor cells appeared about 3 hours after treatment. Six to 12 hours following the treatment, the mitotic rate increased showing 6 to 7 per cent. Although the cells at prophase underwent no morphologic change, the relative number of prophase cells prominently decreased. The division of almost all cells was blocked at metaphase, since 95.3 per cent of mitotic cells was metaphasic ones. They displayed striking abnormalities as shown in Figures 3 and 4. There was a pronounced decrease in number of cells at anaphase and telophase. The observations may be well understood by reference to Table 1.

In samples taken after 12 to 24 hours exposure the vast majority of the HeLa cells were completely disintegrated showing vacuolization of nuclei (Figs. 1 and 2)



Figs. 1–11: Photographs showing effects of TAAO on HeLa cells *in vitro*. Dose: 1–8, 500γ, and 9–11, 2000γ. 1 and 2, nuclear vacuolization, twelve hours after the treatment. ×1000. 3, general view of metaphasic cell. ×600. 4, metaphasic cell 24 hours after treatment. ×600. (1–4, from toluidine blue preparation). 5–8, successive changes of the nucleolus (toluidine blue). ×400. 5, control cells. 6, 12 hours after treatment. 7, 24 hours after treatment. 8, 48 hours after treatment. 9–11, phase microscope photomicrographs showing the behavior of unaffected residual cells in HeLa cells following TAAO-treatment. Note the transformation in shape of cells into the original epithelial type. 9, 12 hours after treatment. 10, 4 days after treatment. 11, 10 days after treatment. **Figs. 12–15**: The mitotic figures of MTK-sarcoma III tumor cells after TAAO application. ×800. 12, prophase, MGG stain. 13, metaphase, squashed and dahlia stain. 14, anaphase, MGG stain. 15, ana-telophase, MGG stain.

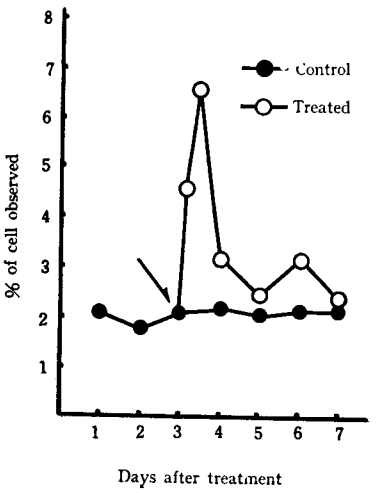


Fig. 16.

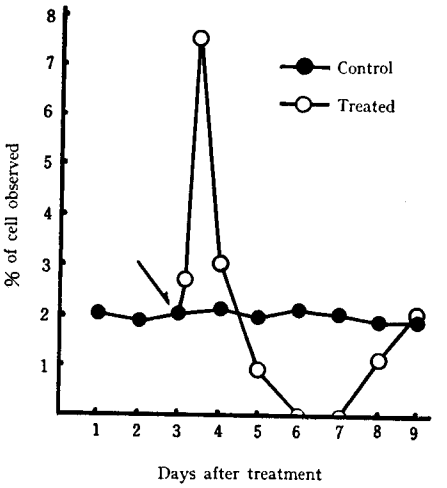


Fig. 17.

Fig. 16. Mitotic frequency of HeLa cells before and after 500γ TAAO treatment.
Fig. 17. Mitotic frequency of HeLa cells after 1000γ TAAO treatment. Arrow indicates the injection of TAAO.

Table 1. Mitotic frequency (%) of HeLa cells in each mitotic stage after 500γ TAAO treatment.

Hours \ Stage	Pro	Meta	Ana	Telo
Cont.	22.2	66.8	22.7	8.3
1	8.4	83.5	8.1	0
3	16.1	83.9	0	0
6	7.9	91.0	0.1	0
12	0.5	97.5	0	0
24	11.3	88.7	0	0
48	17.0	83.0	0	0
72	8.7	90.5	0.2	0.6

Table 2. Mitotic frequency (%) of HeLa cells in each mitotic stage after 1000γ TAAO treatment.

Hours \ Stage	Pro	Meta	Ana	Telo
Cont.	19.1	64.3	11.9	4.7
1	7.4	92.6	0	0
3	16.7	83.3	0	0
6	4.7	95.3	0	0
12	4.7	95.3	0	0
24	8.2	91.8	0	0
48	12.5	87.5	0	0
—	—	—	—	—
144	17.1	61.0	10.7	11.2

and loss of cytoplasmic stainability with MGG or toluidine blue as characteristic features. By this time, mitotic cells showed a rapid decrease, counting about 3 per cent in 24 hours samples. Metaphasic cells showed about 90 per cent in occurrence, whilst the remainder were prophasic cells (Table 1). By 48 hours after the application of the drug, most affected cells had undergone degeneration. In those samples there occurred a certain number of cells, rounded and small in outline, which had persisted alive. They were characterized by nucleoli of rod-like appearance with an intense stainability to the dyes. The metaphasic cells were still blocked, but prophase cells showed a frequency similar to that of untreated cells. By 72 to 96 hours after treatment, almost all cells had attained regular growth, and cells showing metaphase block decreased in number. The increase in number of cells at ana-telophase after treatment took place in a condition similar to the control.

Noticeable is a change in the number of nucleoli after TAAO treatment (Fig. 18). After treatment the cells with one nucleolus increased gradually: 6 hours after treatment 30 per cent of cells observed were those with one nucleolus, while in 24-

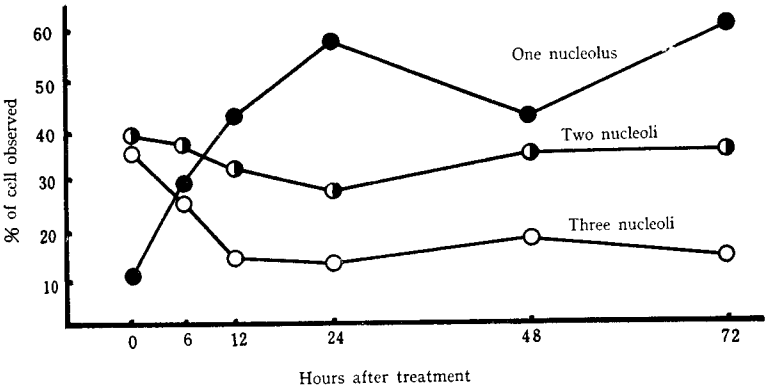


Fig. 18. Number of nucleolus after 500γ TAAO treatment, showing that the cells with one nucleolus increased soon after the tieatment, while those with two or three nucleoli decreased.

hour samples such cells provided 60 per cents. In 72 hour-samples, such cells showed more or less than 60 per cent. On the other hand, there was slight decrease in the cells with three or two nucleoli (Fig. 18).

Of particular interest is the fact that cells of spherical outline remained alive 3 to 4 days even after the application of 2000γ TAAO. There is a possibility that the proliferation of those residual cells may form the primary source of renewed growth. In order to examine the above possibility, a phase microscopy study was undertaken on the HeLa cells which had been treated with 2000γ TAAO for 24

hours. After treatment, cultures were observed under the phase microscope continuously during a period of 24 hours. The results of this observation seemed to support the above view as follows: the affected cells, round in outline and relatively small in size, showed a gradual elongation with a slight movement. Six days later, most cells became epithelial in general appearance characteristic to healthy HeLa cells. Then they displayed signs of following mitotic activity. Examples are presented in Figures 9 to 11.

II. *Effects of TAAO on MTK-sarcoma III and EM-tumor*

Table 3 provides data on the life span of tumor-bearing animals after application of TAAO at various dosages. The average length of survival time was about 9 days for MTK-sarcoma III-bearing rats, and about 10 days for EM-tumor-bearing mice.

Table 3. Life span to tumor-bearing animals after the application of TAAO.

Tumor	Animal (Strain)	Dose	Sex	Life span Average (days)	Range (days)
MTK-III	Rat (Wistar)	Control	4(♀3, ♂1)	8-10	8.8
		5000γ/kg	4(♀3, ♂1)	10-11	10.3
		10000 〃	5(♀4, ♂1)	9-13	9.8
		50000 〃	2(♀1, ♂1)	5-6	5.5
EM-tumor	Mouse (EM)	Control	10(♀4, ♂6)	8-11	9.5
		1000γ/kg	5(♀2, ♂3)	10-12	11.5
		2000 〃	5(♀1, ♂4)	10-12	11.0
		5000 〃	5(♀3, ♂2)	6-7	6.4

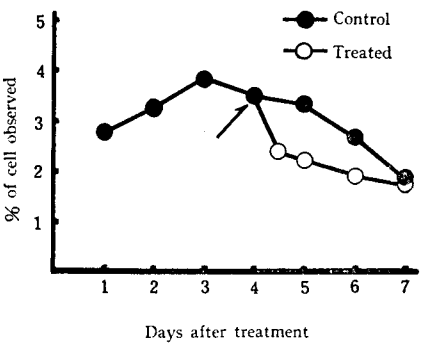


Fig. 19.

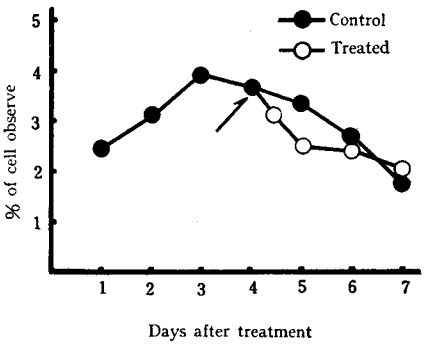


Fig. 20.

Fig. 19. Mitotic frequency in MTK-sarcoma III after 5000γ treatment.
Fig. 20. Mitotic frequency in MTK-sarcoma III after 10000γ treatment.
An arrow indicates the injection of the agent.

The maximum tolerated dosage level was judged on the basis of elongation of survival time and loss of body weight of animals under examination. The range of concentration of the compound used as noted above was 5000γ to 50000γ per kg of body weight. The dosage of 5000γ for rats and 2000γ for mice showed no marked toxicity to tumor-bearing animals, with a slight elongation of survival time resulted (10.3 days for rats and 11.0 days for mice).

It is apparent from the data presented in Table 3 that the agent was relatively non-effective or only slightly effective against tumor cells at 5000γ for rats and at 2000γ for mice. As shown in Figures 19 and 20, the mitotic frequency was similar to that of non-treated samples. After the application of 5000γ TAAO to MTK-sarcoma III, the number of mitotic cells slightly decreased. As shown in Figures 12 to 15, those mitotic cells evidenced no detectable abnormality.

Discussion

Cytological studies on the effects of chemicals and radiations upon non-malignant and malignant cells have been carried out extensively by many investigators in the field of experimental cancer chemotherapy (Biesele 1958, 1961, Makino 1957, 1959). There are many chemicals known as mitotic poisons (Biesele 1958). Among the effective agents, some have produced spindle inhibition of the colchicine type and some others have exerted effect similar to that of ionizing radiation causing chromosomal bridging and breakage. It was concluded by Makino (1957) and his co-workers that the temporary regression of tumor growth was induced to a greater or less degree as a result of damage to a large number of the tumor cells. However, some of the tumor cells remained unaffected by the action of chemicals and furnished the primary source for renewed malignant growth.

A seven-carbon-ring compound, 1-thia-3-azazulan-2-one (TAAO), used as the antitumor agent in this study was found in tissue culture to be one of the mitotic poisons. The most interesting observation was that affected HeLa cells became blocked at metaphase after treatment. The affected metaphasic cells showed no spindle body. To the author's knowledge, there are many papers which report a similar cytological feature on colchicine- and ribonuclease-treatment (Firket *et al.* 1955, Brachet 1957). It is probable that normal cell division occurs at prophase and that the spindle formation is affected at pro-metaphase by the agent. As a result the affected metaphase cells lose their function to divide and consequently they are blocked at metaphase.

Miura *et al.* (1958) reported on the basis of biochemical studies that TAAO inhibited RNA metabolism of rat liver as much as 69 per cent, and showed a somewhat weaker effect on that of silk glands which are known to be one of the animal organs with the largest RNA content. It seems probable that TAAO exerts on RNA metabolism in spindle, and that prophasic cells are blocked at metaphase.

The action of TAAO was further remarkable by the change occurring in the nucleolus of HeLa cells: the polymorphic nucleolus transformed into a spherical

shape. The change in number of nucleoli was also interesting: the cells with one nucleolus increased, and those with two or three nucleoli slightly decreased soon after treatment. Stainability of the nucleoli with basic dyes remained unaltered. It is apparent that the transformation in shape and number of the nucleoli may take place in association with the change in the metabolism of nucleolar RNA.

After treatment with TAAO, there occurred a temporary reduction of growth of the HeLa cells to a greater or less degree as a result of the damage to most of the cells, but there was no complete disintegration of the tumor culture. After the removal of the agent the tumor cells showed proliferation. It seems highly probable that the renewed proliferation of the HeLa cells is caused by multiplication of residual stem-cell which remained alive without being damaged by the agent. Similar features were reported by Hori and Sasaki (1958) in a spindle-cell sarcoma *in vitro* following treatment of Sarkomycin.

Following TAAO application on the MTK-sarcoma III and the EM-tumor, the tumor-bearing animals showed no remarkable prolongation of their life span beyond the average survival days.

Reported evidence has shown that generally tumor-inhibitors are similar in effect between *in vivo* and *in vitro* materials (Sasaki 1956, Hori and Sasaki 1958, Awa 1959, Kobayashi 1959, 1960). However, Ormsbee *et al.* (1947) stated that a given agent might have certain effects in tissue culture that are not observed in the living animals. The results of this study with TAAO indicate that the cytological effects differ between *in vitro* and *in vivo* materials. Ormsbee *et al.* (1947, 1948) stated that the differences in effects between *in vivo* and *in vitro* materials may possibly be due to minute variations in quantities of the given agent.

Summary

The effects of 1-thia-3-azazulan-2-one were cytologically studied with HeLa cells, MTK-sarcoma III of rats and EM-tumor of mice.

It was found that after the application of this drug, affected HeLa cells were blocked at metaphase and showed change in number and shape of nucleoli. The compound furnished no visible effect on the growth of the MTK-sarcoma III of rats and EM-tumor of mice. It seems likely that this drug has different effects upon *in vivo* and *in vitro* materials.

References

Abercrombie, M. 1961. The basis of the locomotory behaviour of fibroblasts. Exp. Cell Res. Suppl. **8**: 188–198.

Awa, A. 1959. Tne cytological effect of chemicals on tumors, IX. Cytological response of the MTK-sarcoma III cells to Carzinophilin and thio-TEPA. Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **14**: 140–148.

Biesele, J.J. 1954. Assay of carcinolytic and carcinostatic agents. Ann. New York Acad. Sci. **58**: 1129–1145.

Biesele, J.J. 1958. Mitotic poisons and the cancer problem. Elsevier, Amsterdam.

Biesele, J.J. 1961. On the mechanism of antimitotic action as studied with cancer cells and antimetabolites. Pathologie-Biologie **9**: 466–473.

Brachet, J. 1957. Biochemical Cytology. Academic Press Inc. New York.

Firket, H., Chèvremont-Comhaire, S. and Chèvremont, M. 1955. Action of ribonuclease on living cells *in vitro* and synthesis of deoxyribonucleic acid. Nature **176**: 1075.

Hori, S.H. and Sasaki, M. 1959. Cytochemical studies on tumor cells, IV. Some cytochemical observations on the effects of Sarkomycin and Carzinophilin on normal and neoplastic cells *in vitro*. Cytologia **24**: 259–271.

Hsu, T.C., Milofsky, L. and Fuerst, R. 1958. Studies on 4-aminopyrazolo (3, 4-d pyrimidine): Reversal of cellular damage in the HeLa strain. Texas Rep. Biol. Med. **16**: 472–478.

Jacobson, W. and Webb, M. 1952. The two types of nucleoproteins during mitosis. Exp. Cell. Res. **3**: 163–183.

Kobayashi, J. 1959. Some cytological and cytochemical studies on effects of "Mitomycin-X" on the spindle cell sarcoma *in vitro*. (A preliminary report). Jap. Jour. Genet. **34**: 344–350.

Kobayashi, J. 1960. The cytological effect of chemicals on tumors, X. On the effect of Mitomycin C on tumor cells of two animal tumors. Cytologia **25**: 280–288.

Lison, L. 1953. Histochimie et cytochimie animales. Gauthier-Villars, Paris.

Makino, S. 1957. The chromosome cytology of the ascites tumors of rats, with special reference to the concept of the stemline cells. Intern. Rev. Cytol. **6**: 25–84.

Makino, S. 1959. The role of tumor stem-cells in regrowth of the tumor following drastic applications. Acta **15**: 196–198.

Miura, Y., Noguchi, T. and Takeyama, S. 1958. Études sur la Biosynthèse de l'Acide Ribonucléique. Pathol. Biologie **34**: 1869–1878.

Miura, Y. and Shigematsu, A. 1958. Effects des azulénoïdes sur la division cellulaire chez les Orusins. Compt. rend. Soc. Biol. **152**: 221.

Ormsbee, R.A., Cornman, I. and Berger, R.E. 1947. Effect of podophyllin on tumor cells in tissue culture. Proc. Soc. Exp. Biol. Med. **66**: 586–590.

Ormsbee, R.A. and Cornman, I. 1948. The place of tissue culture in a cancer chemotherapy screening program. Cancer Research **8**: 384–385.

Sasaki, M. 1956. The cytological effect of chemicals on ascites sarcomas, V. Effects of Cortisone and Sarkomycin on the MTK-sarcoma II. Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **12**: 433–441.

Takeyama, S., Ito, H. and Miura, Y. 1958. Fibroin synthesis and ribonucleic acid metabolism in the silk gland. Biochimica et Biophysica Acta **30**: 233–243.