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THE EFFECT OF VITAMIN B_6 ON GROWTH OF TUMOR

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ABSTRACT

Attempts have been made to clarify the effect of pyridoxine deficiency as well as its overdosis on growth of transplantable tumors in mouse and rat.

The growth of transplantable solid tumor in pyridoxine deficient animals was remarkably suppressed while that of ascites tumor was not. The tumor bearing host was administered with a large amounts of pyridoxine and pyridoxal phosphate resulting in no effect on either tumor growth or survival time of tumor bearing host. The incorporation of leucine into S-180 ascites tumor cells under pyridoxine deficiency was similar to that in pyridoxine added condition, *in vitro*. In contrast, the efflux of leucine from pyridoxine deficient tumor cells increasd in comparison with that of control tumor cells. Although there were no changes in ultrastructure of tumor cells, the concentration ability of amino acid and protein synthesis from amino acid were decreased in pyridoxine deficient cells.

In conclusion, the present investigation is further support for a role of pyridoxine in tumor growth.

I. INTRODUCTION

After Bischoff[1] and Kline[2] first reported in 1943 that the growth of the tumor transplanted to Vitamin B_6 deficient animal was remarkably suppressed, experimental studies on Vitamin B_6 and the growth of tumor have since been made chiefly by Shapiro, Rosen, Mihich, and Nichol. Namely, discussions were made about the growth of tumor when it was transplanted to the experimental animal[3][4], fed on Vitamin B_6 deficient diet or to the animal administered with the Vitamin B_6 antagonist[5][6], the carcinogenicity when a carcinogenic substance was given to the above mentioned animals[7][8], and the immune reaction against the tumor tissue in the Vitamin B_6 deficient animal[9].

However, it has not always been clear whether the suppression of tumor growth was caused by direct influence of B_6-deficiency on the metabolism of tumor cells or whether the suppression was caused by a change in the biological interaction between tumor and host. The author confirmed that the growth of tumor which had been transplanted to the B_6-deficient animal was remarkably suppressed. The experiment was performed in order to clarify the mechanism

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on the basis of cytological consideration for the behavior of the transplanted tumor in the state of B_6 excess or the metabolic influence of B_6-deficiency on the tumor cells.

II. MATERIALS AND METHODS

Experimental animal: Male mice of the ddN strain weighing 15~20 g and male rats of the Wistar strain and of the Donryu strain weighing 40~50 g were used.

Method of breading: Mice were given diet of the composition shown in (Table 1) according to the method of Mihich and others[10], and the rats were put on diet of the composition shown in (Table 2) according to Yamada's method[11] ad libitum with water. Pyridoxine was removed from the diet in the B_6-deficient group. The experimental animals were maintained individually at room temperature of 20~24°C in wire net cages to prevent feces contamination.

TABLE 1. Compositon of Diet for Mous

Basal Diet	
Saccharose	72%
Casein	20%
Corn Oil	4%
Salt Mixture (McCallum)	4%
Vitamins per 100 g Basal Diet	
Thiamin-HCl	1 mg
Riboflavin	1 mg
Pyridoxine-HCl	1 mg
Niacin	4 mg
Ca-Pantothenate	6 mg
Inositol	15 mg
PABA	20 mg
Biotin	20 μg
Folic Acid	20 μg
Choline-Cl	200 mg
Cod Liver Oil	500 mg

TABLE 2. Composition of Diet for Rat

Basal Diet	
Glucose	66%
Casein	20%
Cotton Seed Oil	10%
Salt Mixture (McCallum)	4%
Vitamins per 100 g Basal Diet	
Thiamin-HCl	0.4 mg
Riboflavin	0.6 mg
Pyridoxine-HCl	0.6 mg
Niacin	2.0 mg
Ca-Pantothenate	2.0 mg
Inositol	30 mg
PABA	10 mg
Folic Acid	50 μg
Biotin	50 μg
Vit. B_{12}	10 μg
Choline-Cl	100 mg
Cod Liver Oil	500 mg

Transplanted tumor and method of transplantation: Sarcoma-180 and Yoshida sarcoma were obtained from the Research Institute for Tuberculosis and Leprosy, Tohoku University, and Walker carcinosarcoma-256 from the Research Institute of Takeda Pharmaceutical Co. The S-180 of both solid and ascitic type were successively transplanted every 7~8 days in the mice of ddN strain fed with Oriental solid diet, Yoshida sarcoma was transplanted every 5~6 days to the rats of Donryu strain, and Walker carcinosarcoma every 10 days to the

rats of Wistar strain. A definite number of ascitic tumor cells suspended in Ringer's solution was transplanted intraperitoneally, while a small piece of the solid type tumor was inoculated subcutaneously into the axilla using trocar.

Measurement of the tumor growth: S-180 tumor was measured by the long and short axis using a caliper, and the mean is called the average tumor diameter. Walker carcinosarcoma-256 was measured by the wet weight. In the case of the ascitic tumor, a total amount of the ascites was collected by the mesenteric reflux with heparin-added Ringer's solution of known volume from the peritoneal cavity of the tumor bearing animal to determine the amount of ascites and the total number of the cells[12]. Moreover, the ascites was washed more than 3 times with the Ringer's solution by low centrifugation (500~800 r.p.m.), in order to collect only the tumor cells after removing erythrocytes contamination. Finally, they were centrifuged at 3,000 r.p.m. for 10 minutes to eliminate as much water as possible for the determination of wet weight.

Value of liver transaminase activity: The liver of the experimental animal was homogenized with cold water to determine GOT and GPT per wet weight of the liver according to Reitman-Frankel's method[13].

Measurement of total Vitamin B_1: This was done according to Fujiwara's[14] Parmtit-thiochrome method.

Measurement of total Vitamin B_2: The measurement was made according to Yagi's[15] Lumiflavin-fluorescent method.

Measurement of total Vitamin B_6: The measurement was done by Atkin-Fukui's[16] microbial method using Saccharomyces Carlsbergensis. The measurement of Vitamin B complex was carried out by adding a known amount of Vitamin, calculating the recovery rate with correction.

B_6-deficient tumor cells and non B_6-deficient tumor cells: As stated above, male mice of ddN strain weighing 15~20 g were fed for 2 weeks with B_6-deficient and non-B_6-deficient diet. And then 10×10^6 of S-180 tumor cells which were washed with Ringer's solution were introduced intraperitoneally into each group of the animals. At the 9th day after the transplantation, the ascites was collected from a few mice of the B_6-deficient group. The tumor cells were counted after washing 3 times by centrifugation in heparin added Krebs-Ringer phosphate solution for the removal of erythrocytes, and were used for experiments as the B_6-deficient tumor cells. Furthermore, the ascites was taken from 2 to 3 mice of the non-B_6-deficient group, and the tumor cells were pooled to use for experiments as non-B_6-deficient tumor cells.

Measurement of the radioactive amino acids:
DL-leucine-1-^{14}C (specific activity 66 mCi/mM)

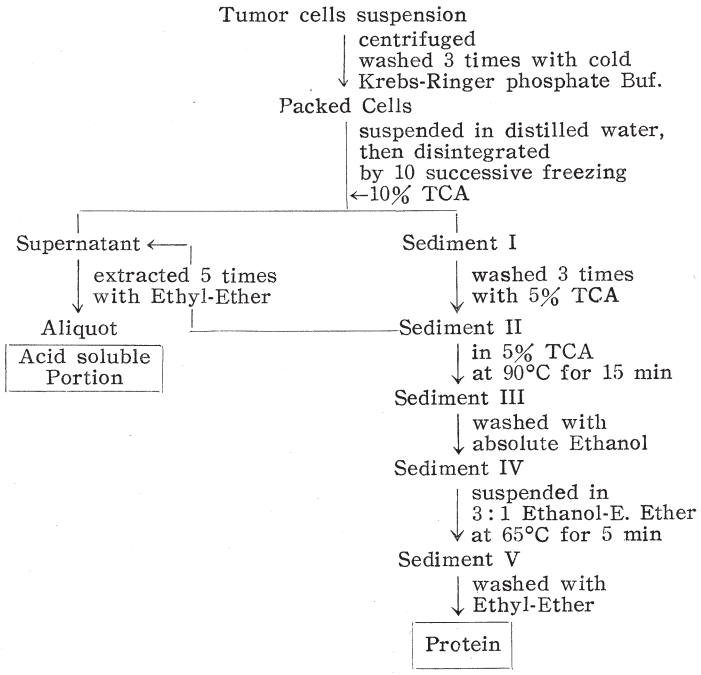
L-leucine-^{14}C (u) (S.A. 155 mCi/mM)

L-Serine-3-^{14}C (S.A. 20.4 mCi/mM)

The three kinds of radioactive amino acids as mentioned above were used for experiment and obtained from the Radiochemical Centre (RCC). The determination of the radioactivity was made by using 2π gas flow counter.

Extraction of intracellular protein fraction and acid soluble fraction: After the tumor cells were lyophylized according to Rabinowitz's[17] method, the protein and acid soluble fractions were extracted by the method shown in (Table 3).

TABLE 3



Determination of the amount of protein: The determination was carried out by Lowry's[18] Folin-Phenol method.

Extraction of DNA within tumor cell and method of the determination of DNA: The tumor cells were washed several times with cold Krebs-Ringer phosphate buffer, added with 0.25 M saccharose, then homogenized and centrifuged at 600 G for 10 minutes to remove the cytoplasmic components. The sediment was then washed twice with 0.3 N perchloric acid to remove the acid soluble fraction. Later the sediment, which was suspended in 4 ml of 10% saline containing phenol red, was neutralized with NaOH.

The nucleic acid component was extracted in the solution of 10% saline in the boiling water bath for 25 minutes and then centrifuged. 2 volume of 95% ethanol was added to the supernatant which was left standing overnight when sodium salt of nucleic acid was precipitated. After the centrifugation, the sediment was extracted at 50°C for 5 minutes with a 3 : 1 mixture of ethanol and ether. This mixture was heated at 80°C for 15 minutes with 3 ml of 0.1 N NaOH to hydrolize RNA. After cooling, 1 N HCl was added to this solution for final concentration of 0.1 N, which was precipitated to extract DNA fraction. The DNA fraction was washed with 95% ethanol and dissolved in 1 ml of 0.1 N NH_4OH. A part of the mixture was used for determination of DNA and the other was dried on the aluminium sample disk for the determination of radioactivity[19].

Determination of the amount of DNA: This was done by Diphenylamine method[20].

Electron-microscopic observation: The specimen was immediately put in the 1% Osmium tetroxide solution which was dissolved in the phosphate buffer of pH 7.4 and fixed for 20 minutes. After fixation it was dehydrated with ethanol and embedded in Epon to prepare the electron microscopic section using LKB Ultra tome. This section was stained with saturated acetic aciduranium solution according to Reynold's method[21] and then photographed under JEM-5 type electron-microscope (initial magnification $\times 2{,}000 - \times 5{,}000$).

III. EXPERIMENTAL RESULTS

1) Growth of the tumors transplanted to Vitamin B_6 deficient animal

The effects on the growth of the tumor in transplanting various kinds of tumors to the experimental animals deficient in Vitamin B_6, have been studied by Mihich and his colleagues extensively. The author has conducted experiments to reconfirm these facts.

a) Sarcoma 180 (Solid)

Male mice of ddN strain with 18~20 g of the first body weight were fed with the diet having the composition as shown in Table 1 which is divided into the B_6-deficient group and non-deficient group. The mean body weight (mean of 10 animals) in each group are as shown in (Fig. 1).

In the non-deficient group, an increase of 32% in the body weight was found in 3 weeks, while in the deficient group the increase in the body weight ceased after one week and even a decrease was noted in the 3rd week. After 3 weeks, the both groups of mice were transplanted with S-180 subcutaneously in the axilla, and the average tumor size was measured in course of time for observing the growth of tumor (Fig. 2). In the B_6-deficient group the growth of tumor was noted to be markedly suppressed in comparison with the non-

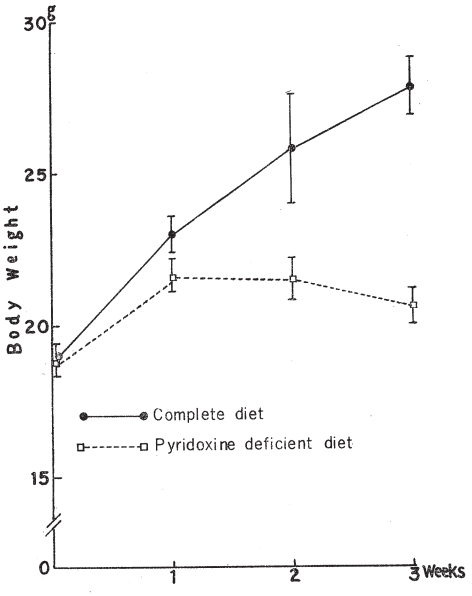


FIG. 1

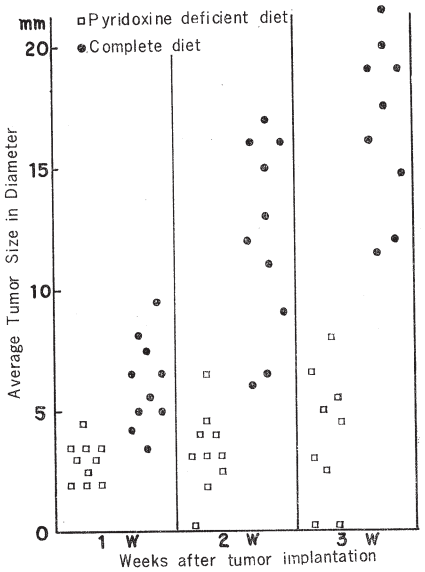


FIG. 2

FIG. 1. Effect of pyridoxine deficiency on mouse body weight.
FIG. 2. Effect of pyridoxine deficiency on the growth of S-180 solid.

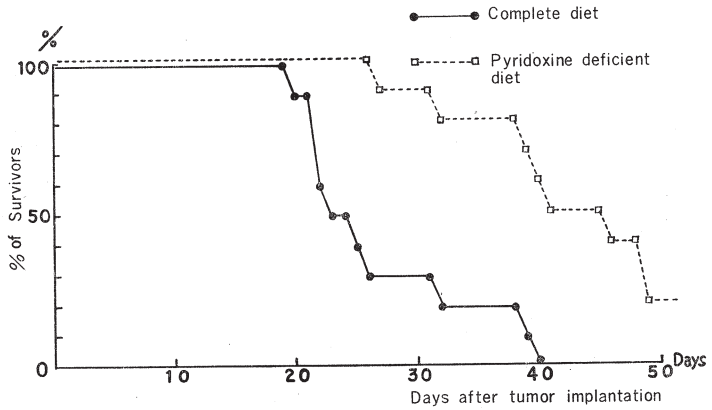


FIG. 3. Surviving rate of mice bearing S-180 (solid).

deficient group. The cases with non-palpable tumor (complete regression) was noted in B_6-deficient group.

Moreover, the survival rate of the tumor-bearing mice in the both groups reported as (Fig. 3). The prolongation of survival period was clearly recognized as 100% of death was observed during 20 to 40 days after transplantation of tumor in the non-deficient group, while 40% of survival rate over 45 days

TABLE 4. Vitamin Content and Transaminase Activity of Liver bearing S-180 Solid

	Contents of Vitamin B				Activity of Transaminase		
	No.	B_1 ($\mu g/g$)	B_2 ($\mu g/g$)	B_6 ($\mu g/g$)	No.	GOT unit	GPT unit
Complete Diet	1	8.04	22.8	9.02	1	147×1000/G of w.w.	37×1000/G of w.w.
	2	9.16	32.7	6.60	2	110	34
	3	7.30	19.6	6.52	3	129	43
	4	7.88	33.9	7.40	4	65	28
	5	—	—	6.92	5	99	32
	Mean	8.09	27.3	7.29	Mean	110	34.8
Pyridoxine deficient Diet	1	8.92	32.3	3.88	1	78	19
	2	7.40	39.6	2.64	2	89	21
	3	8.08	24.1	2.51	3	73	25
	4	5.80	18.5	2.40	4	62	16
	5		29.5	4.65	5	87	24
	Mean	7.55	28.8	3.36	Mean	77.6	21

in the B_6-deficient group.

In order to clarify whether or not the suppression of tumor growth as stated above was characteristic in the B_6-deficiency or was only due to the reduced intake of total calorie, each group of five tumor-bearing mice at 8 days after being transplanted were chosen and exsanguined after decapitation to determine total amounts of Vitamin B_1, B_2, and B_6 and the value of transaminase activity in the liver (Table 4). As shown in the table, there were no difference between total amount of B_1 and B_2 of both groups, while total amount of B_6 was found to be decrease considerably in the B_6-deficient group, and the activity value of liver transaminase exhibited about 29% decrease in GOT* and about 40% decrease in GPT* in the deficient group compared with the non-deficient group.

Therefore, the tumor-bearing mice which had been fed with B_6-deficient diet were in definitely B_6-deficient state.

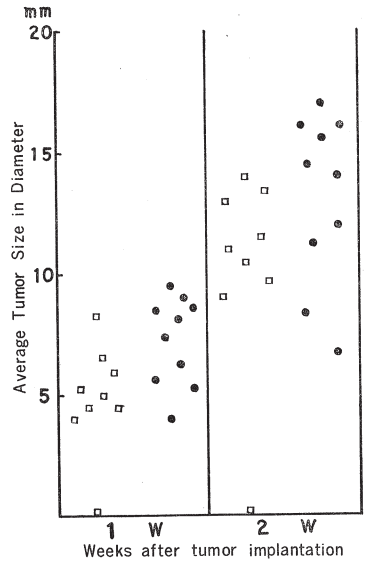
Next, the effect of low protein diet on the growth of S-180 solid tumor was simultaneously observed. Mice of ddN strain were fed with the diet containing 4% of casein for 2 weeks as same as in the experiment of the B_6-deficiency (the total calorie was supplemented with glucose). There is no increase in the body weight of mice which was fed with low protein diet containing 4% of casein; the mice weighing 16.3 g (on the average) before the feeding weighed 16.1 g (on the average) 2 weeks after. In addition, the growth of tumor was not always suppressed in comparison with that of the control group. With exception only one case was observed with complete

* GOT: Glutamic oxaloacetic transaminase.
GPT: Glutamic pyruvic transaminase.

regression (Fig. 4).

b) S-180 (Ascites)

As described above, after having been fed with B_6-deficient and non-B_6-deficient diet for 3 weeks, both groups of mice were transplanted intraperitoneally with suspension of 10×10^6 ascitic tumor cells in 0.2 ml of Ringer's solution. The survival period of the tumor-bearing animal and the amount of ascites, total count of tumor cells, and wet weight of tumor cells in the ascites at the 9th day after the transplantation were obtained (Fig. 5), (Table 5). As shown in (Fig. 5), no difference of survival period was noted between the B_6-deficient and non-deficient groups, and in all mice of the deficient group the ascites was observed, and complete regression as encountered in the S-180 solid was not observed. As demonstrated in (Table 5), in the B_6-deficient group, amount of ascites, weight of tumor cells, and total count of tumor cells were decreased about 40%, 60%, and 57% respectively in comparison with those in the non-deficient group. Generally, total amount of ascites was much more reduced in comparison with the count of ascitic tumor cells. Furthermore, the same experiment as described above was carried out by transplantation of $10 \times 10^4 \sim 10 \times 10^3$ of tumor cells. However, when transplanted with less tumor cells, the transplantation rate decreased



●: Complete diet □: Casein 4%
Mice were fed for 2 weeks prior to tumor implantation.

FIG. 4. Effect of low casein diet on growth of S-180 solid.

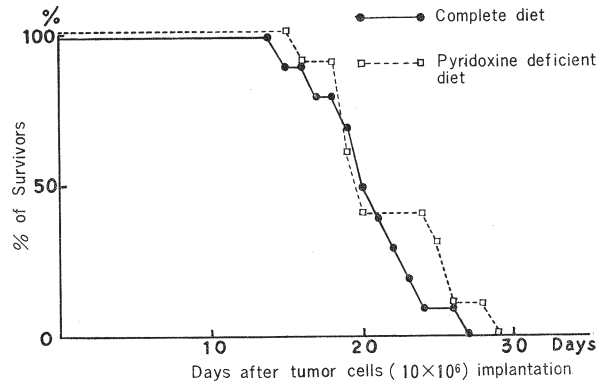


FIG. 5. Surviving rate of mice bearing S-180 (ascites).

TABLE 5. Effect of Pyridoxine Deficiency on Growth of S-180 Ascites

	Body Weight (g)	Volume of Ascites (ml)	Weight of Ascites Cells (g)	Total Number of Ascites Cells ($\times 10^6$)
Pyridoxine Deficient Diet	21.9	3.3	1.21	498
	17.8	2.4	1.10	543
	23.0	2.8	1.28	451
	21.5	2.1	0.79	266
	24.9	2.2	0.95	354
	21.7	1.9	1.04	584
	25.7	1.3	0.60	336
	22.5	2.0	1.01	490
Mean	22.4	2.25	0.99	435
Complete Diet	32.5	5.3	1.87	761
	31.2	5.9	2.07	683
	30.4	5.7	2.36	805
	31.2	5.2	2.02	813
	31.9	5.3	2.51	745
	30.8	6.4	2.18	872
Mean	31.3	5.63	2.17	763

9 days after tumor cell (10×10^6) inoculation.

even in the non-deficient group. While the low transplantation rate in the deficient group was due to the high mortality rate which merely resulted from the B_6-deficiency of the experimental animal, there was no certain tendency in this aspect.

c) Walker carcinosarcoma-256

Male rats of Wistar strain with 50~60 g of the initial body weight, were divided into B_6-deficient and non-deficient group, and they were fed with the

TABLE 6. Body, Spleen, Liver and Tumor (Walker carcinosarcoma 256) Weight of Rat Fed on Pyridoxine Deficient and Complete Diet, Respectively

Pyridoxine Deficient Diet					Complete Diet				
Rat No.	Body weight (g)	Spleen (g)	Liver (g)	Tumor (mg)	Rat No.	Body weight (g)	Spleen (g)	Liver (g)	Tumor (mg)
1	124	0.28	4.95	28	1	164	0.40	5.50	580
2	136	0.31	5.10	30	2	162	0.32	5.45	810
3	130	0.30	4.32	29	3	140	0.28	4.60	2550
4	133	0.51	5.10	13	4	160	0.35	4.72	1300
5	131	0.35	4.60	55	5	160	0.30	5.40	600
6	98	0.16	3.50	19	6	154	0.25	4.54	650
7	104	0.25	4.15	36	7	148	0.30	5.10	720
8	132	0.41	4.60	44	8	146	0.41	5.80	2350
9	122	0.21	4.20	0	9	150	0.32	4.35	3100
10	128	0.29	4.75	36	10	138	0.25	3.90	1340
Mean	124±12	0.31±0.09	4.51±0.48	32±11	Mean	152±9	0.32±0.06	4.84±0.52	1400±88

diet of the composition as shown in Table 2 for 4 weeks. Each was transplanted subcutaneously in the abdominal wall with Walker carcinosarcoma-256. Ten days later, they were exsanguined after decapitation to remove the liver, spleen, and tumor, and each were weighed for wet weight (Table 6). In the B_6-deficient group, no significant difference was observed in the weights of the liver and spleen in comparison with those in the non-deficient group, but the weight of tumor tissue was reduced to about 1/40 with marked suppression of tumor growth.

d) Yoshida's sarcoma ascites

Male rats of Donryu strain with 40 to 60 g of initial body weight, were divided into both B_6-deficient and non-deficient groups by the above method, and they were fed for 4 weeks. Each of them was transplanted intraperitoneally with 10×10^5 of Yoshida's sarcoma cells to observe the survival rate. No significant difference was noted in the survival period between both deficient and non-deficient groups (Fig. 6). In the deficient group all the animals exhibited retention of ascites and died of tumor between 9 to 16 days later.

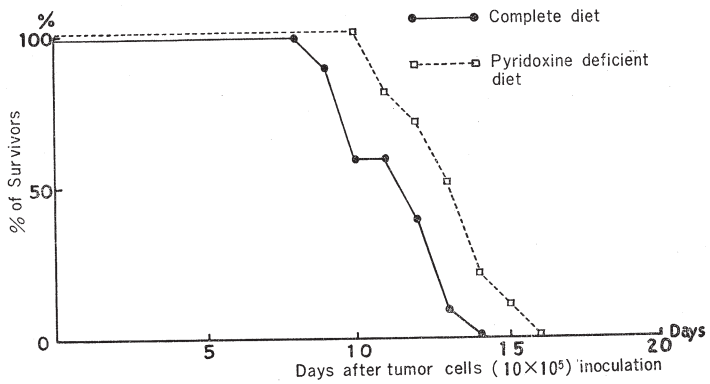


FIG. 6. Surviving rate of rats bearing Yoshida sarcoma ascites.

Brief Summary

After mice of ddN strain were transplanted with S-180 (solid type and ascitic type), rats of Wistar strain with Walker carcinosarcoma, and rats of Donryu strain with Yoshida's sarcoma, the growth of tumors were observed for its extent in each of B_6-deficient and non-deficient groups. The growth of solid tumor transplanted subcutaneously with S-180 solid type or Walker carcinosarcoma, was markedly suppressed by the B_6-deficiency, while tumor of ascitic type such as S-180 ascites or Yoshida's sarcoma was noted to be suppressed for their growth not so remarkably as the solid tumor was. The survival rate of tumor bearing animals showed no significant difference between the both groups. Moreover, mice of ddN strain in the B_6-deficient group which

had been fed with the compound diet, made by the author, for a certain period exhibited a decrease in the total amount of B_6 in the liver and the activity value of transminase which confirm that the mice was in B_6 deficient state. At the same time total amount of B_1 and B_2 in the liver showed no difference between the B_6-deficient and non-deficient groups, and the tumor growth of low protein mice which had been fed with 4% of Casein was not so remarkably suppressed in comparison with that of B_6 deficient group.

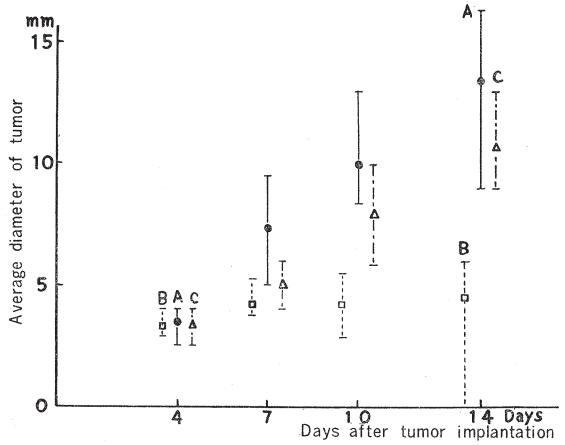
From these facts, it could not be considered that such suppression of tumor growth in the B_6 deficient animal was merely caused by a slightly decrease in the total calorie resulted from the reduced intake of diet in the host.

2) Effect of Vitamin B_6 administration on the growth of transplanted tumor

As stated above, it has been proved that the growth of the transplanted tumor S-180 solid was markedly suppressed by B_6-deficiency in the host. So the following experiment was carried out to examine whether the growth of tumor is affected by a change in the administered doses of Vitamin B_6 or not.

a) Administration of small amounts of Vitamin B_6 (Relative deficiency)

Male mice of ddN strain weighing 17~18 g were divided into 3 groups of non-deficient, relatively deficient and deficient groups of pyridoxine content of 1 mg, 0.1 mg, and 0 mg respectively per 100 g of the compound diet with the composition shown in Table 1. These mice were fed for 2 weeks to observe in course of time the growth of S-180 solid transplanted subcutaneously to each



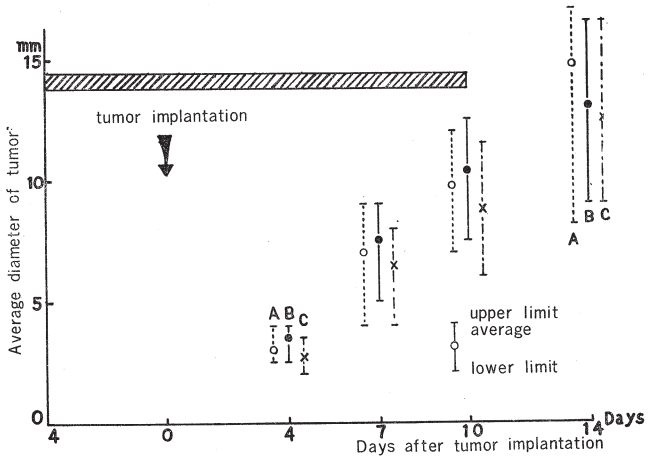
A: Complete diet. B: Pyridoxine deficient diet.
C: Relatively pyridoxine deficient diet.
All diet were given for 2 weeks prior to tumor implantation.

FIG. 7. Effect of pyridoxine deficiency on the growth of S-180 (solid).

axilla (Fig. 7). As indicated in the figure, the deficient group exhibited marked suppression of the growth of tumor as in the above mentioned experiment, and the 2nd week after the transplantation, a complete regression of the tumor was noted. In the relatively deficient group, the growth of tumor was suppressed until one week after the transplantation, but after 10 days it showed similar result as to that seen in the non-deficient group, and there was no complete regression.

b) Administration of large amounts of Vitamin B_6

Mice were fed with the non-deficient diet to be transplanted with S-180 solid at the first week. From 4 days before the transplanatation, the experimental animals were divided into 3 groups, and each group was administered intraperitoneally with a relatively large amounts of PIN* 50 mg per kg of body weight and PAL-P* 50 mg per kg respectively for successive 14 days, the control group being injected intraperitoneally with an equal amount of physiological saline (0.2 ml) for the same period of time. The observation of the growth of tumor made in course of time revealed that there was no noticeable difference in the extent of the tumor growth (Fig. 8) and the survival period of the tumor bearing mice among the three groups of control group and 2 groups administered with a large amount of PIN and PAL-P respectively (Fig. 9).



A○: Group of PIN overdosis. B●: Group of 0.9% NaCl solution.
C×: Group of PAL-P overdosis.

Tumor implantation average diameter of tumor.

FIG. 8. Effect of vitamin B_6 overdosis on the growth of S-180 (solid).

* PIN: Pyridoxine.
PAL-P; Pyridoxal-5'-phosphate,

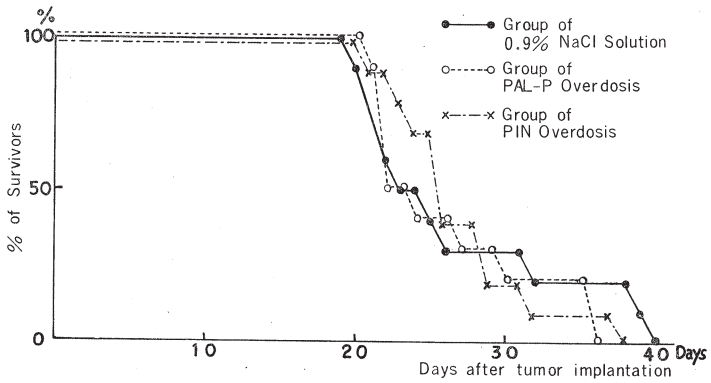


FIG. 9. Surviving rate of mice bearing S-180 (solid). Effect of vitamin B_6 overdosis.

c) Changes in the Vitamin B_6 concentration of liver and tumor of the tumor-bearing mouse after loading with pyridoxine

Mice, which had been fed with non-deficient diet for one week, were transplanted with S-180 solid and 7 days after the transplantation PIN was injected intraperitoneally with the doses of 10 mg per kg of body weight into the tumor-bearing mouse after 24 hours fasting. A total amount of Vitamin B_6 of the liver and tumor at 30, 60, and 120 minutes after injection was measured (Fig. 10). As shown in the figure, in the tumor-bearing mouse loaded with PIN, a total amount of B_6 per g of wet weight of the tumor tissue reached a peak in 30 minutes and gradually decrease, while a total amount of B_6 per g in the liver reached a peak with a much higher concentration in the tumor tissue. However, the increasing rate obtained from a comparison with the concentration per g of wet weight before the administration was 3.5 times at maximum in the liver, but in the tumor it was 5.3 times at maximum. It is presumed that this is because the tumor tissue is much smaller than the liver, and the concentration of B_6 before the administration is much lower

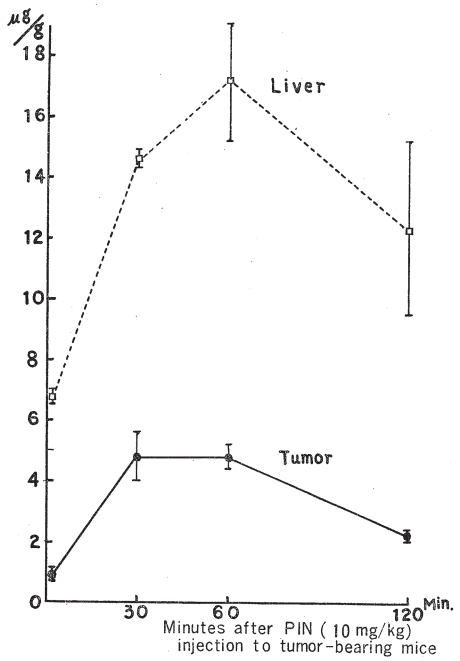


FIG. 10. Total vitamin B_6 content of liver and tumor after pyridoxine injection to S-180 bearing mice.

in the former than in the latter.

Brief Summary

When B_6 was administered to tumor-bearing animals, even a large amount of B_6 could not accelerate the growth of the tumor though it was transferred into the tumor tissue from the blood in the same way as into the liver and no influence was observed on their survival time. Also on the oxperimental result of the relative deficiency it was found that although S-180 required B_6 to some extent for its growth, higher concentration of B_6 had no influence, and the tumor continue to grow naturally.

3) The cytological behavior of the tumor cells in the B_6 deficient circumstance

From the preceding two experiments, it was found that the growth of the tumor was considerably suppressed in the B_6-deficient milieu, because it required Vitamin B_6 to some extent. The growth of the solid tumor should be discussed in relationship of the strain of host, the resistance of the host transplanted and, the surrounding supporting tissue and the proliferation of the blood vessels to the tumor tissue. In order to eliminate such complicated factors, metabolic influence of the B_6-deficient milieu on the tumor cells themselves was examined on the cytological level.

a) Character of S-180 ascites deficient in Vitamin B_6

After mice of ddN strain were fed with B_6-deficient and non-deficient diet as described in the previous chapter (1) b), the animals of the both groups were transplanted intraperitoneally with 10×10^6 of tumor cells of S-180 ascitic type. On the 9th day after the transplantation, the ascites was collected from experimental animals of the both groups. After the number of tumor cells per mm^3 of this ascites was counted, the supernatant of the ascites and tumor cells were centrifuged to be separated, and each being determined for the total amount of B_6 (Fig. 11) and protein content of ascites (Fig. 12). As shown in the figure, the total B_6 content in the tumor cells was reduced to about 1/4 of that in the non-deficient group. Moreover, the total B_6 content of the supernatant of ascites was remarkably reduced to 1/15 in comparison with that of the non-deficient group. But the protein content of the supernatant of the ascites (per dl) and the number of the tumor cells per mm^3 showed no remarkable difference between the B_6 deficient and non-deficient groups.

b) Uptake and efflux of amino acid by S-180 ascitic tumor cells

In B_6-deficient and non-deficient S-180 ascitic tumor cells, the uptake of radioactive amino acids by the tumor cells and the efflux of radioactive amino acids from tumor cells *in vitro* was examined. As mentioned in the chapter of the experimental method, each of equal number of B_6-deficient tumor cells and non-deficient tumor cells was suspended in the Krebs-Ringer phosphate buffer solution (without $CaCl_2$) and was incubated in air at 37°C after being

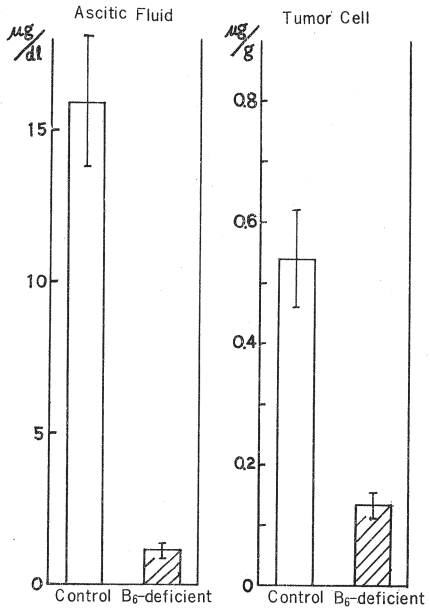


FIG. 11

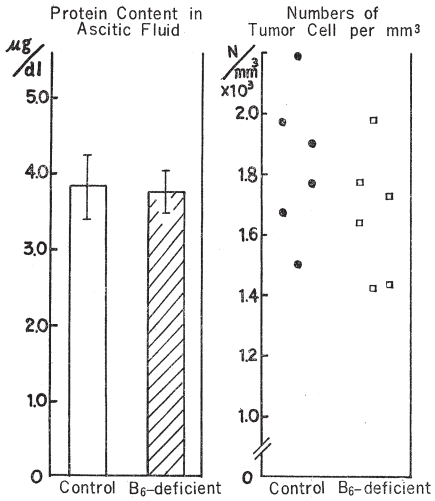
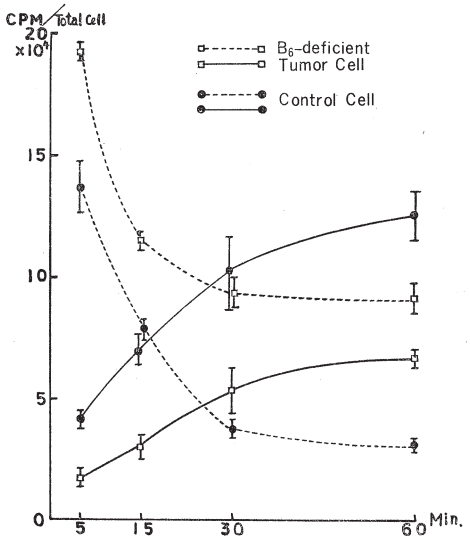


FIG. 12

FIG. 11. Total vitamin B_6 content of S-180 ascites.
FIG. 12. Protein content of S-180 ascites and numbers of tumor cell.



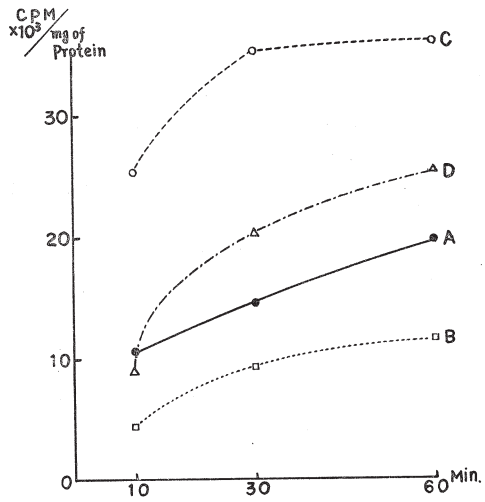
Incubation medium: S-180 tumor cells (10×10^6), DL-leucine-1-^{14}C 5 μci suspended in Krebs-Ringer phosphate buffer pH 7.4 at 37°C.

FIG. 13. Incorporation of amino acid (DL-leucine-1-^{14}C) to acid soluble fraction and protein of S-180 tumor cells.

added with 5 μci of radioactive DL-leucine-1-^{14}C until a total volum of 2 ml.

After incubation for 5, 15, 30, and 60 minutes, the specimen were cooled on the ice immediately, and the protein and the acid-soluble fraction containing the tumor cells were extracted by Rabinowitz's method. The uptake of amino acids into each of them was observed by the measurement of total radioactivity (Fig. 13). The uptake of DL-leucine into the protein of the tumor cell was increased with hours, while that into the acid-soluble fraction within the tumor cell decreased in course of time. In the B_6-deficient tumor cell, the uptake of DL-leucine into the intracellular protein was reduced to about 2/3 of that in the non-deficient tumor cell, but the uptake of DL-leucine into the acid-soluble fraction was rather higher than that of the non-deficient group. It increased about 3 times during 60 minutes incubation.

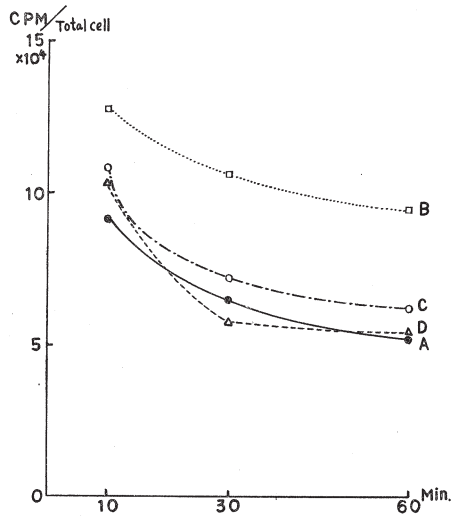
The uptake of L-leucine-^{14}C (u) into the intracellular protein in the B_6-deficient and non-deficient tumor cell was indicated by the specific radioactivity per mg of protein (Fig. 14). In the B_6-deficient tumor cell, the radioactivity per mg of intracellular protein was reduced to about 2/3 of that in the non-deficient tumor cell. Moreover, when the glucose was added to the incubation medium became for final concentration of 0.2%, both the tumor cells showed a marked increase in the uptake of L-leucine into the intracellular protein. The increasing rate was greater in the non-deficient tumor cell (Fig. 14).



A: Control cell B: B_6 deficient cell
C: Glucose added to A D: Glucose added to B

Incubation medium: S-180 ascites tumor cells 10×10^6, L-leucine-^{14}C (u) 2 μci suspended in krebs-ringer phosphate buffer (pH 7.4) at 37°C.

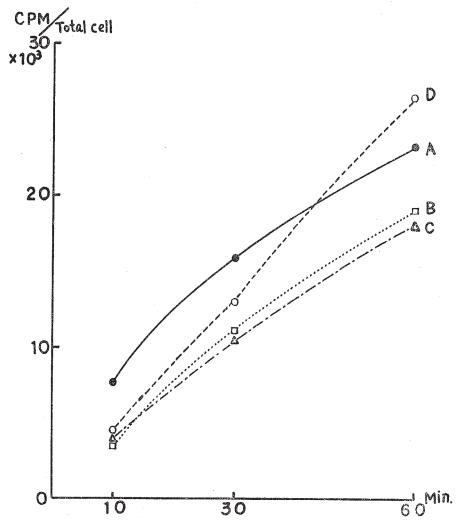
FIG. 14. Amino acid (L-leucine-^{14}C) incorporation to protein of S-180 tumor cells.



A: Control cell
B: B_6 deficient cell
C: 1.0 μM of PAL-P added to B
D: 1.0 μM of PIN added to B

Incubation medium: S-180 ascites tumor cells 10×10^6, L-leucine-^{14}C (u) 2 μci suspended in Krebs-Ringer phosphate buffer (pH 7.4) at 37°C.

FIG. 15. Effect of vitamin B_6 on amino acid (L-leucine) incorporation to acid soluble fraction of S-180 tumor cells.



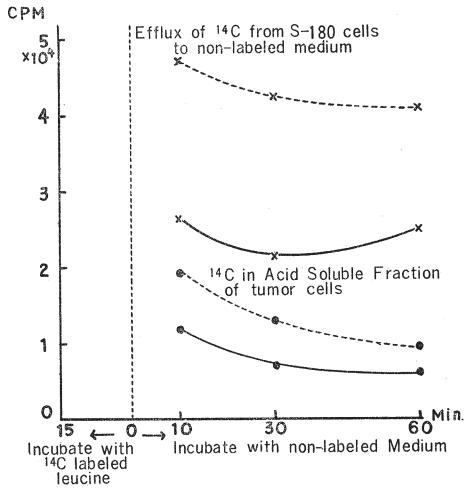
A: Control cell
B: B_6 deficient cell
C: 1.0 μM of PAL-P added to B
D: 1.0 μM of PIN added to B

Incubation medium: S-180 tumor cells 10×10^6, L-leucine-^{14}C (u) 2 μci suspended in Krebs-Ringer phosphate buffer (pH 7.4) at 37°C.

FIG. 16. Effect of vitamin B_6 on amino acid (L-leucine) incorporation to protein of S-180 tumor cells.

An experiment was made to study whether the incorporation of leucine into the intracellular protein of the B_6-deficient tumor cell approach to that of the non-deficient tumor cell in the incubation medium with PIN or PAL-P of various types of Vitamin B_6. The radioactivity of L-leucine-^{14}C (u) in the intracellular acid-soluble fraction was reduced with hours by addition of PAL-P or PIN (Fig. 15). The incorporation of L-leucine-^{14}C into the intracellular protein of the B_6-deficient tumor cell was noted to increase in 60 minutes of incubation by addition of PIN, but slight increase was noted by addition of PAL-P (Fig. 16).

Then, the efflux of radioactive amino acids from tumor cells which once took up the radioactive amino acids was examined. As mentioned above, after taking of the same number (6×10^6) from both B_6-deficient ascitic tumor cells and non-deficient tumor cells, each of them was suspended in 5 ml of the Krebs-Ringer Phosphate buffer (containing 0.2% of glucose) with 4 μc of radioactive L-leucine-^{14}C (u) and was incubated in air at 37°C. After shaking 15 minutes, tumor cells were washed 3 times with ice cold Krebs-Ringer solution



------ with B_6-deficient tumor cells
—— with non-deficient tumor ells
upper: CPM/ml of medium
lower: CPM/total cell

FIG. 17. Efflux of (^{14}C) from S-180 cell.

to eliminate radioactive substance adhering to the surface of cells and were re-suspended in non-radioactive incubation medium. After shaking for 10, 30, and 60 minutes, radioactivity of ^{14}C were counted in the incubation medium excluding radioactive amino acid and in the intracellular acidsoluble fraction (Fig. 17).

Efflux of the radioactive amino acids to medium from B_6-deficient tumor cells was greater than that of non-deficient tumor cells, and the remained radioactivity of ^{14}C simulutaneously observed in the acidsoluble fraction of B_6-deficient tumor cells was higher than that of non-deficient tumor cells.

c) DNA synthesis of the B_6-deficient tumor cell

An experiment was made to examine whether the B_6-deficient tumor cell would show a decrease in the ability of nucleic acid synthesis as it showed *in vitro* in the ability of protein synthesis from L-leucine-^{14}C. Serine was chosen which is an important precursor of purine synthesis in the course of nucleic acid synthesis and also in close relationship to the B_6 enzyme, and its uptake into the nucleic acid within the tumor cell was observed. The B_6 deficient mice and non-deficient mice were transplanted intraperitoneally with 180 ascitic tumor cells, and on the 8th day each of tumor-bearing mice was injected

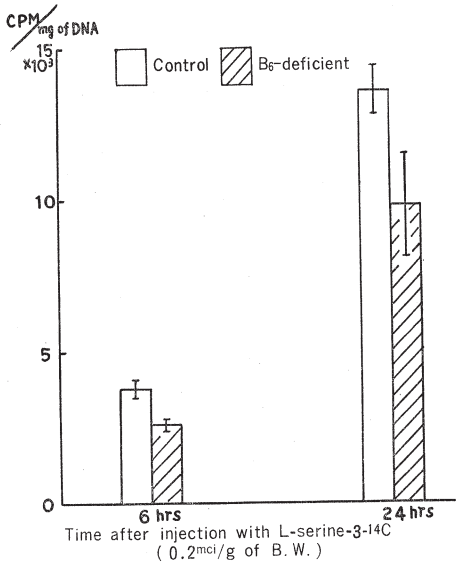


FIG. 18. Incorporation of ^{14}C into DNA of S-180 tumor cells after L-serine-3-^{14}C injection to tumor-bearing mouse.

intraperitoneally 0.2 mCi of L-serine-3-^{14}C per kg of the body weight. At 6 and 24 hours after the injection, the ascites was obtained from the mouse of both groups to collect the tumor cells of which the specific radioactivity per mg of DNA were determined (Fig. 18). Moreover, no radioactivity of ^{14}C was noted in the supernatant of the ascites of the tumor-bearing mouse at 48 hours after the injection of L-serine-3-^{14}C. As shown in Fig. 18, in the B_6 deficient S-180 tumor cell, the uptake of L-serine-3-^{14}C into its DNA decreased to about 28~30% of that in the non-deficient tumor cell.

d) Electronmicroscopic observations of the B_6 deficient S-180 ascitic tumor cell

In order to know whether the decrease of the ability to synthesize protein and nucleic acid in the B_6 deficient S-180 tumor cell as noted in the preceding chapter b) and c) was merely due to the degeneration of the tumor cell or due to any morphological change of the cell, electronmicroscopic observations were made on the intracellular change of S-180 tumor cell placed under the B_6-deficient environment. At 8 days after the transplantation of the S-180 ascites tumor cell into the B_6 deficient and non-deficient mice, the ascitic tumor cells were taken from the tumor-bearing mice of the both groups to be fixed immediately. Observations of the ultra-structure of the non-deficient S-180 ascites tumor cell revealed characteristics as shown in (Photo. b), the marked invagination of the nucleus, hypertrophy of the nucleolus, a decrease in the numbers of mitochondria and endoplasmic reticulums, and an increase in free ribosomes. Beside, the formation of microvilli (Photo. b), protrusion of the cell surface (Photo. b), fairly developed Goldi apparatus (Photo. d), and lipoid granules (Photo. c) within the cytoplasm were noted. These characteristics, however, were generally observed not only in the S-180 ascites tumor cell, but also in the other kind of tumor cell. Then, comparative observations were made on the ultra-structure of the B_6 deficient S-180 tumor cell with that of the non-deficient tumor cell (Photo. e, f, g, h). In the nucleus of tumor cell, nodifference was noted in the shape of nuclear membrane, nucleolus, and nucleoplasm between both cells. In the cytoplasm, examinations of microvilli formation, protrusions of the cell surface, mitochondria, Goldi apparatus, free ribosome, endoplasmic reticulums, and lipoid granules revealed no marked difference between both cells in the quantitative and qualitative aspects. Besides, markedly degenerated cells did not exhibit so much quantitative difference between the both groups.

Brief summary

With the S-180 ascitic tumor cells, investigation were made to clarify how the B_6 deficiency would influence on the tumor cell itself by using the method of intracellular amino acid uptake in the functional aspect and using electron-microscopic observation in the morphological aspect. In the case of transplantation of tumor cells to the B_6-deficient animal the amount of Vitamin B_6

within the tumor cell was decreased, but in the supernatant of the ascites, which could be called the liquid sopporting tissue, was remarkably decreased. The equal number of the B_6 deficient tumor cells and the non-deficient tumor cells were taken to be incubated *in vitro* with the radioactive amino acid (leucine), and the both cells were compared for the amino acid uptake into the acid-soluble fraction and the protein fraction. In this test, the B_6 deficient tumor cell, the amino acid uptake of the intracellular acid-soluble fraction increased, but that of the protein fraction decreased in comparison with non-deficient tumor cell. And also, the efflux of radioactive amino acid from tumor cell which took it up increased greater in B_6 deficient tumor cell than in non-deficient tumor cell.

From the above result, it is considered that the ability to concentrate amino acids and to synthesize protein in the B_6 deficient tumor cell would decrease.

Furthermore, in case of adding of Vitamin B_6 to the B_6 deficient tumor cell *in vitro*, both PIN and PAL-P decreased the amount of amino acids in the intracellular acid-soluble fraction, but the uptake of amino acids into protein fraction increased to the extent observed in the non-deficient tumor cell only when added with PIN. However, it was found that the influence of adding with Vitamin B_6 *in vitro* on the ability of protein synthesis within the tumor cell did not appear by certain hours. Namely there exists lag time until PIN display the effect.

The incorporation of serine-3-^{14}C into the nucleic acid of the B_6 deficient S-180 tumor cell is decreased to some extent in comparison with that observed in the non-deficient tumor cell. From this fact, it is acknowledged that the tumor cell exhibited a decrease in the ability to synthesize purine in the B_6 deficient environment.

The following question is raised whether the decrease in the abilities of protein and purine synthesis shown by such B_6 deficient tumor cells is related to a change in the ultra-structure within the cell. Therefore, the ultra-structure of the both cells, mainly free ribosomes, mitochondria, and cell surface, which are considered to have close relationship to intracellular metabolism, were observed by using electronmicroscope. As a result, no particular change in the ultrastructure was noted qualitatively and quantitatively as far as the author experienced. At present time, these functional changes of B_6-deficient tumor cell could not be observed morphologically even by electronmicroscopic study.

IV. DISCUSSION

Mihich, *et al.*[4] studied extensively the growth of tumor in experimental animals which were subjected to Vitamin B_6 deficiency and to which various types of tumor had been transplanted. They transplanted Murphy-Sturm lymphosarcoma and Walker carcinosarcoma 256 to albino rats of the Sprague-

Dowley strain; Ehrlich carcinosarcoma solid, Ehrlich ascitic carcinoma clone 2 and Sarcoma 180 to Swiss mice of HaICR stock; Ridgway osteogenic sarcoma and Leukemias L 4946 to mice of AKR stock, Adenocarcinoma 755 to mice of C 57 BL/6 stock, and Leukemias L-1210 to mice of C 3 H stock. They observed the development of various types of tumor and the survival rate of tumor-bearing hosts. As a result, they reported that in the case of transplantation of seven types of tumor to mice and one type of tumor to albino rats, the development of tumor had been inhibited remarkably in the B_6-deficient group, and the survival period had been prolonged in all the host animals except those to which the types of tumor of ascitic origin had been transplanted. The present author transplanted S-180 solid and S-180 Ascites to mice of ddN stock, Walker carcinosarcoma 256 to albino rats of Wister strain, and Yoshida sarcoma to albino rats of Donryu strain in two group of laboratory animals, B_6-deficient and non-deficient, then observed the development of tumor and the survival rate of tumor-bearing hosts.

It was made clear that in the case of the subcutaneous-nodule type of Walker carcinosarcoma and S-180 solid, the development of tumor had been inhibited remarkably and the survival period prolonged in the host animals in the B_6-deficient group. However, it was found that in the case of the ascitic type of Yoshida sarcoma and S-180 ascites, the development of tumor was inhibited in the B_6-deficient group, although it was not so remarkable as in the case of tumor of subcutaneous-nodule type, and the survival rate was not prolonged among the host animals of this group. These experimental results, except those obtained from Walker carcinosarcoma, were essentially the same as those reported by Mihich, *et al.*

In addition, Mihich, *et al.* recognized that there were differences in degree of transplanted tumor in B_6-deficient animals among the types of tumor and the species or strains of laboratory animals serving as hosts. They ascribed these findings to the fact that there were differences in nature among the types of tumor transplanted and in severity of B_6-deficiency among the species and strains of animals used for experiment. It is true that there are differences in effect of B_6 deficiency among the species and strains of laboratory animals, as mentioned by Lyon[22] and Fenton, *et al.*[23] What explanation can be made for the difference in development observed between S-180 solid and S-180 ascites in mice of the same strain which had been confirmed to be suffering from B_6-deficiency of the same degree and to which the same type of tumor had been transplanted? White[24] made an extensive review of literature and mentioned that the development of transplantable tumor in experimental animals was retarded, and the incubation period between the transplantation and the initiation of development was prolonged on account of restriction put upon uptake of total nutritional calorie and each nutrient. He stated that in those animals, the development of tumor was generally considered to have been

inhibited, as compared with that in a group of animals which had been fed without any restriction on nutrition during the same period, and maintained that inhibition upon the development of tumor tissue transplanted particularly in the subcutaneous region might be induced by such disturbances upon the formation of blood vessels and stroma of the subcutaneous connective tissue as caused by the restriction of nutrition. Besides, Greenberg[25] reported that arteriosclerosis was apt to occur in B_6-deficient animals which exhibited thickening of the endothelium with mucous-like substance seen on histological examination. From these findings, it is considered that ascitic tumor cells might be sufficiently in contact with nutritive materials, regardless of the contact with the surrounding supporting tissue and the hyperplasia of blood vessels, and that the development of tumor, therefore, might not be inhibited in the transplanted tumor of ascitic type so remarkably as in that of subcutaneous-nodule type in B_6-deficient animals.

Then, an attempt must be made to determine whether the inhibition upon development of transplanted tumor in B_6-deficient animals is induced by same effect of B_6-deficiency upon the state of the tumor-bearing host or by some changes brought about to the metabolism of the transplanted tumor cells themselves.

In discussing the development of transplanted tumor, it is natural to take such immunological and biological reaction as induced between tumor and host into consideration. Mihich[9] mentioned that whole-body X-ray irradiation performed before transplantation reduced the rate of complete regression of S-180 tumor and the effect of inhibition of development of this tumor transplanted in B_6-deficient mice, and suggested that there might be an increase in the immunological resistance of the host to tumor or a decrease in such antibody as promoting the development of tumor in the body of B_6-deficient animals. Recently, Mihich[26] has also reported that the rate of complete regression of S-180 tumor caused by B_6-deficiency was reduced in the mice which had been subjected to thymectomy immediately after parturition. He attributed the effect of B_6-deficiency to the inhibition of development of S-180 tumor, and the complete regression of this tumor to a reduction in the immunological resistance of the host. On the other hand, he reported that the S-180/B_6 tumor strain, which was resistant to B_6 deficiency and could develop under B_6-deficient conditions, had been produced by repeating the transplantation of tumor cells to B_6-deficient mice[27].

Mice to which S-180 had been transplanted were not strictly pure-bred ones from the immunological point of view. Any antibody promoting the development of tumor failed to be demonstrated. From these results, it is impossible to affirm that the effect of inhibition upon the development of tumor in B_6-deficient animals is derived exclusively from the resistance of the host. In general, laboratory animals kept on B_6-deficiency show a decrease not only

in circulating antibody, but also in ability to reject the tissue transplanted heterogenously. This fact has been made clear by a number of experiments performed[28)29)]. In addition, regression is induced in the thymus by B_6 deficiency[30)]. Taking all the results mentioned above into consideration, it seems to be doubtful that those laboratory animals in which B_6 deficiency has reduced the immunological resistance have come to possess a resistance enhanced exclusively to the tumor transplanted.

There is another problem in the case of tumor cells kept on B_6-deficient conditions. What change occurs in the tumor cells themselves? The results of experiments conducted by the present author indicate that the amount of Vitamin B_6 was definitely reduced in S-180 ascitic tumor cells transplanted to B_6-deficient mice. Accordingly, it has been confirmed that B_6 deficiency can be induced in S-180 ascitic tumor cells, as well as in other tissues of the B_6-deficient animal and bacteria cultivated in B_6-deficient medium.

Christensen[31)] was the first to carry out biochemical studies on changes in tumor cells themselves under B_6-deficient conditions. He observed Ehrlich ascitic tumor cells which were derived from B_6-deficient mice and found that they decreased in intracellular uptake of glycine *in vitro* and in ability to concentrate it, and that the intracellular transfer and the ability to concentrate were restored when pyridoxal had been added to those cells. After that, many papers[32)33)] have been published on the mechanism of intracellular transmission of Vitamin B_6, particularly pyridoxal and glycine, in tumor cells and bacteria.

All of them affirm that pyridoxal plays an important role in the mechanism of transport of amino acid (particularly glycine). In the author's experiment, transport of leucine into S-180 ascitic tumors was not inhibited under B_6-deficient conditions, and the trnsport of leucine was restored only when pyridoxine had been added. Besides, the leucine taken into B_6-deficient tumor cells was released in much larger amount than that taken into non-B_6-deficient tumor cells. These results suggest that Vitamin B_6 does not increase the transport of amino acid to tumor cell but decrease the release of amino acid from cells, as reported by Heiz[34)].

Holden assumed that this mechanism might take place because B_6-deficiency induced some change in the cell membrane, for example, thining of membrane which bring changes in the osmotic pressure[35)].

Then, the author examined B_6-deficient S-180 ascitic tumor cells in detail by electron microscopy, but there was no difference in the fine structure of the cell or the properties of the surface of the cell between B_6-deficient and non-B_6-deficient tumor cells.

The author has the opinion that the decrease in ability to concentrate occurs functionally rather than the result of the decrease in transport of amino acid into B_6-deficient tumor cells.

Then, another question raised why a larger amount of leucine is contained

in the acid-soluble fraction of the B_6-deficient tumor cell than in that of the non-B_6-deficient tumor cell. It seems to be the reason for this is that there is a decrease in the transfer of leucine into such fractions other than the acid-soluble ones as protein, nucleic acid, and lipoprotein. In fact, the uptake of leucine by the intracellular protein was reduced and the ability of the protein synthesis decreased in B_6-deficient S-180 ascitic tumor cells in the present investigation. These results may support the reason mentioned above.

Hence, the author presumes taht in tumor cells a decrease may be first induced functionally in the ability of intracellular concentration of amino acid and in the ability of protein synthesis by B_6-deficiency, and that organic changes may be caused in those cells themselves with process in severity of B_6 deficiency.

Finally, discussion is made to determine whether or not the inhibition upon the development of transplanted tumor cells under B_6-deficient conditions is brought about by such changes in metabolism as induced by B_6-deficiency in the host. It is considered that the laboratory animal yields the metabolite not existed physiologically by B_6-deficiency, and these substances act to tumor cell as cytotoxic substances, but these substances have not been demonstrated yet.

According to Wiss and Weber[36], when laboratory animals are subjected to B_6 deficiency, the following metabolic disturbances are mainly induced in their body; hindrance in the absorption of amino acid through the intestinal tract, increase in the excretion of amino acid into the urine, and hindrance in protein synthesis caused by disturbances in amino acid synthesis and mutural conversion of amino acid components due to a decrease in activity of B_6 enzyme.

Accordingly, in the body of the B_6-deficient animal, there occur an increase in glycine and a decrease in serine as the changes in the composition of amino acid in the plasma, hepatic tissue, and muscular tissue[37]. It is reported by Wentworth[38] that Vitamin B_6-deficiency cause a decrease in water and an increase in fat as changes in the composition of the organism. When the decrease in water in the body was compared between B_6-deficient and non-B_6-deficient mice to both of which had been transplanted S-180 ascitic tumor cells, there were no marked differences in the amount of protein or the number of cells contained in a unit quantity (in ml) of ascites between the two groups of mice, but the total amount of ascites decreased more distinctly in the B_6-deficient mice.

In addition, hindrance in purine nucleic acid synthesis was also recognized in B_6-deficient animals[39]. The author injected serine-3-^{14}C into B_6-deficient and non-B_6-deficient tumor bearing animals to determine the uptake of tumor cells into DNA. As a result, it was proved that the incorporation of amino acid by tumor cells into DNA was lower in the B_6-deficient animal than in the control ones. There must have been some metabolic changes in the body of

the B_6-deficient animals or in the tumor cells themselves which induced this result. It is quite certain, however, that there is a reduction in the ability of purine synthesis of tumor in B_6-deficient animals.

From the findings mentioned above, it is not difficult to presume that the development of tumor may be affected to some extent by such metabolic changes as induced in the body of the B_6-deficient animal. However, generally speaking, tumor cells proliferate, surmounting the nutritional conditions of the host or metabolic changes in the host. In the author's investigation with B_6-deficient mice bearing S-180 Ascites, the decrease in Vitamin B_6 content was much more remarkable in the ascites than in the tumor cells. These results suggest the presence of autonomicity in the development of tumor.

The mechanism of inhibition upon the development of tumor (mainly S-180) in B_6-deficient animals was discussed from the immunological and cytochemical point of view, as well as from the viewpoint of metabolism in the host.

V. CONCLUSION

Using mice and rats, the effect of Vitamin B_6 on the growth of transplanted tumor and characteristics of ascitic tumor cells in Vitamin B_6 deficiency were examined cytologically, and the following conclusion was obtained.

1) In the case of tumor transplantation to Vitamin B_6 deficient animals, the growth of the tumor forming the subcutaneous nodule was markedly suppressed, the survival time of the tumor bearing host also was extended. The ascitic tumor was noted to be suppressed for the growth, but the suppression was not so remarkable as the subcutaneous type without any noticeable extension of the survival time of the host.

2) When the experimental animals loaded with a large amount of Vitamin B_6 were transplanted with the tumor, its growth is not promoted, showing a characteristics of the tumor itself.

3) The Vitamin B_6 deficient ascitic tumor cells do not exhibit any remarkable change in the fine structure of the cells, but show functionally decreases in the ability of intracellular concentration of amino acids and in the power of protein synthesis.

From the above results it may be concluded that the tumor cells themselves require a certain amount of Vitamin B_6, but the mechanism of inhibition upon the growth of tumor in B_6-deficient animals is so complicated that it is impossible to discuss it with a single factor in mind.

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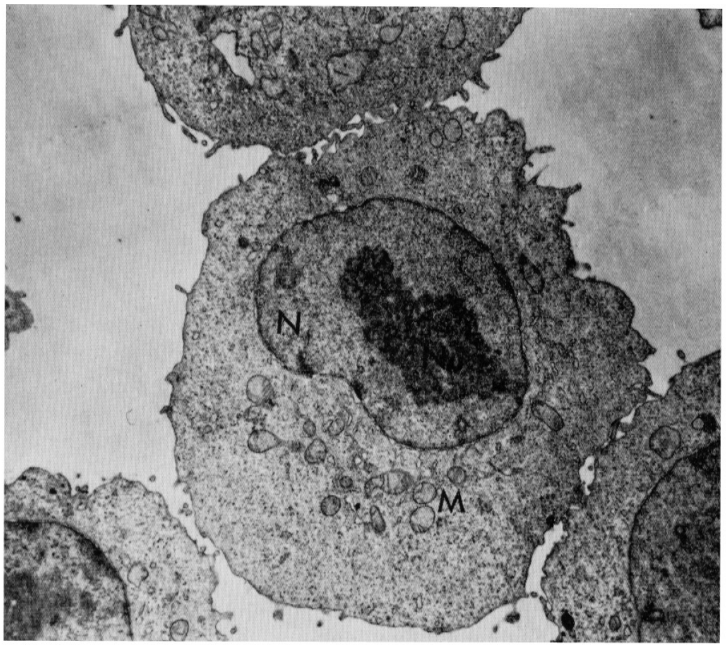
EXPLANATION OF PHOTO.

N : Nucleus
Nu : Nucleolus
Inv. : Invagination of nucleus
M : Mitochondria
G : Goldi apparatus
ER : Endoplasmic reticulum
LG : Lipoid granule
V : Vesicule
mVi: Microvilli
Pro : Protrusion of cytoplasm

PHOTO. a, b, c, d: Electron micrograph of S-180 ascites cells obtained from non-B_6-deficient mouse. 8 days after tumor implantation.

PHOTO. e, f, g, h: Electron micrograph of S-180 ascites cells obtained from B_6-deficient mouse. 8 days after tumor implantation.



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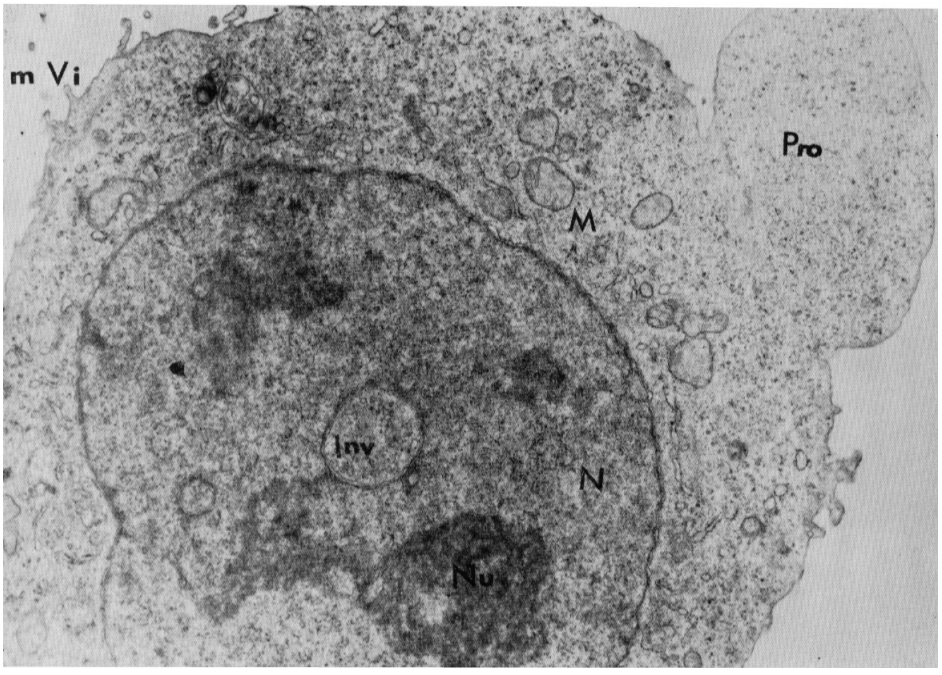

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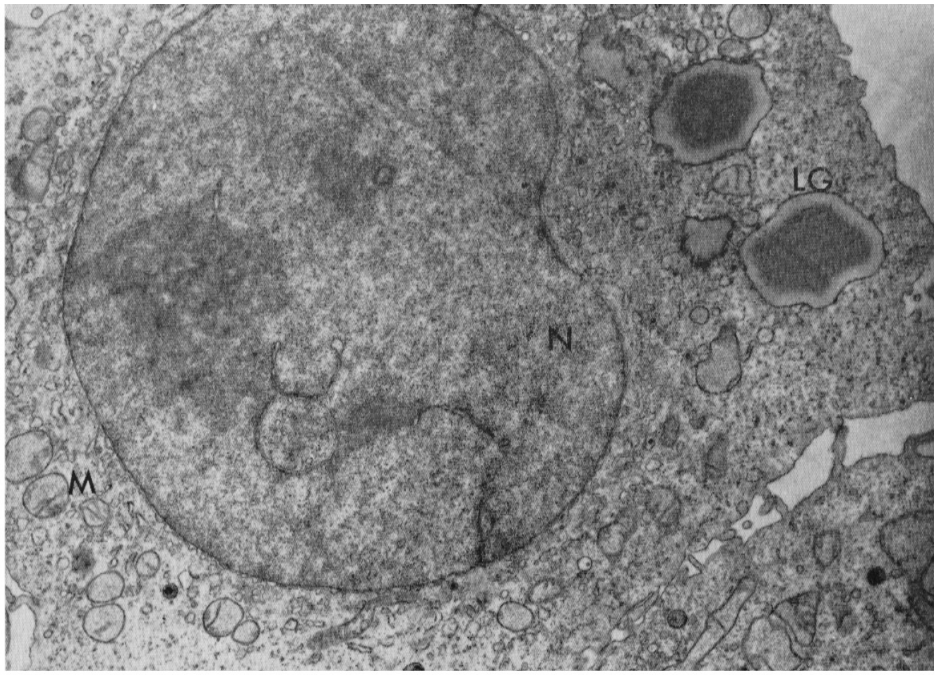

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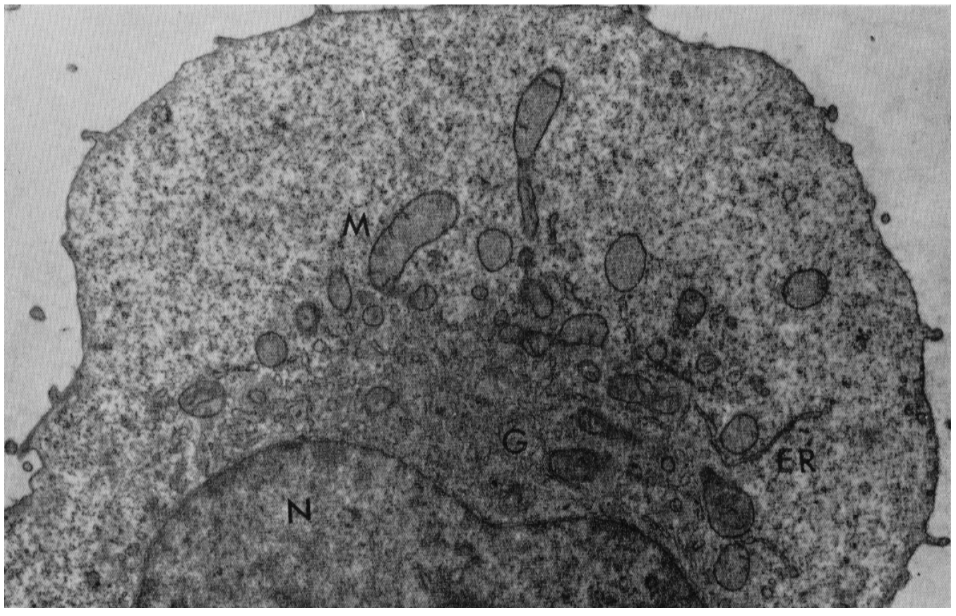

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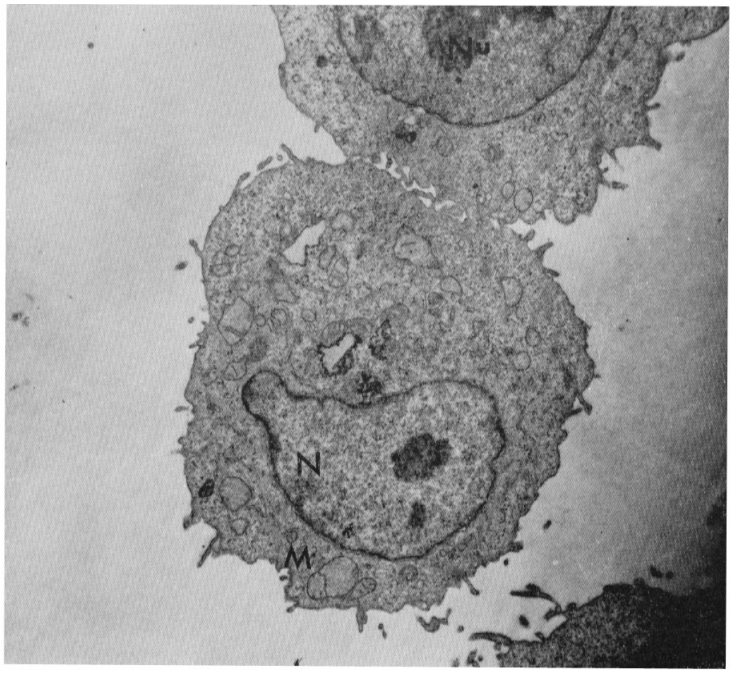

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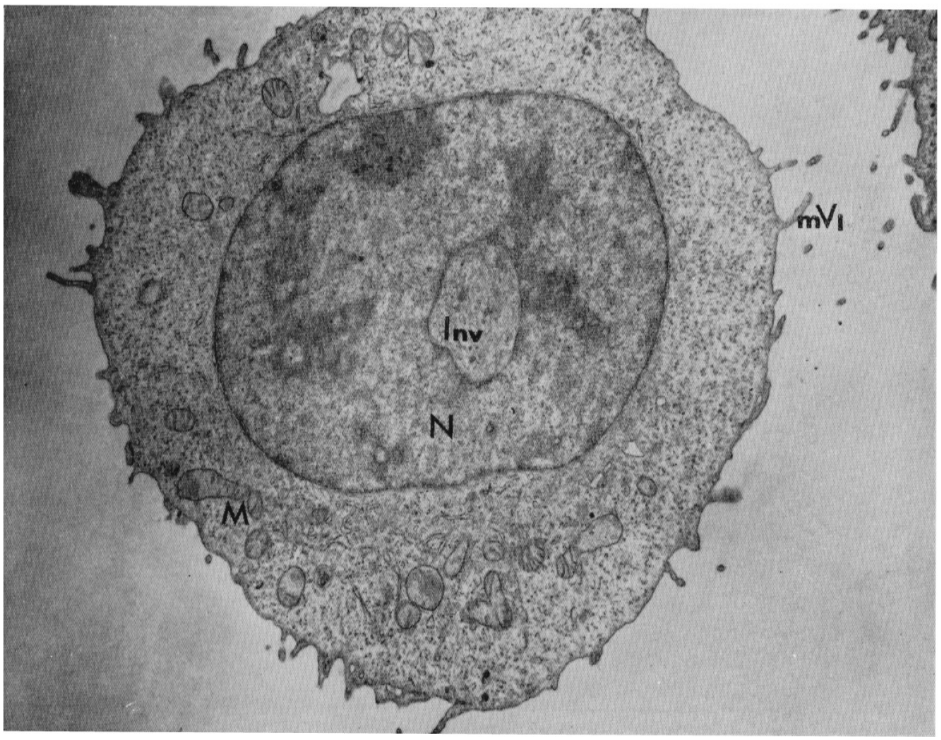

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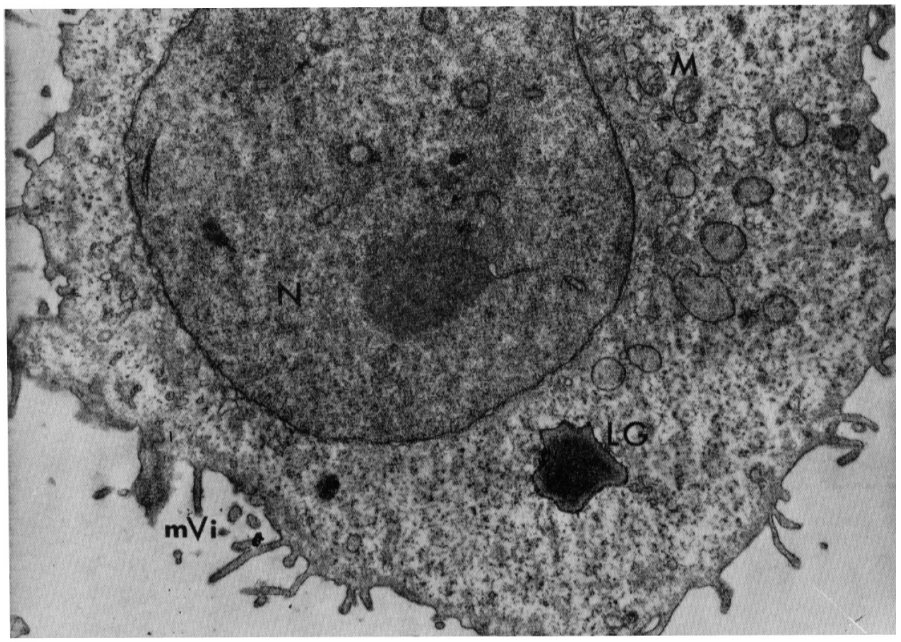

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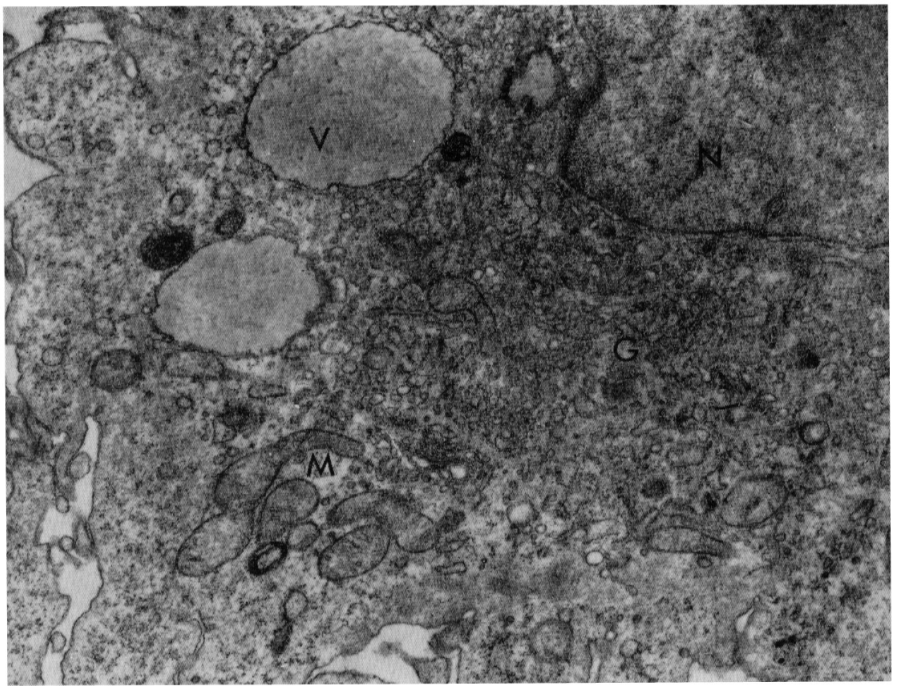

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