

## STUDIES ON THE VARIATION OF SERUM SIALIC ACID AND HEXOSAMINE CONTENTS IN THE SPRAGUE- DAWLEY RATS WITH THE EXPERIMENTAL BONE TUMOR\*

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### ABSTRACT

Variations of serum sialic acid and total hexosamine (glucosamine and galactosamine) levels were investigated in rats of the Sprague-Dawley strain for clarification as follows:

- (1) Physiological variations of these levels by age in normal rats.
- (2) Variations of these levels during the growth of tumor after intramedullary implantation of Walker carcinosarcoma 256; relationship between these values and treatments; and the changes of these values according to the relapse and/or metastasis in the treated rats.

As a result, the values of serum sialic acid and of total hexosamine showed a tendency of gradual increase by age. Serum sialic acid and total hexosamine levels and ratio of glucosamine to galactosamine showed significant increase with the growth of the implanted tumor. After treatments these values returned to normal ranges, but these values were elevated again when recurrence and/or metastasis occurred. Therefore, it may be valuable in observing the response to therapy and in prognosis, to determine these values. There were positive correlation coefficients between the wet weight of tumor masses transplanted subcutaneously and the serum levels of sialic acid and also between the levels of serum sialic acid and total hexosamine in this study. The high contents of total hexosamine were caused to a large extent by the changes of glucosamine contents and to a less extent by the changes of galactosamine levels. Galactosamine, which was found much more than glucosamine in bony tissues, was not released into blood by lesions of the bone substrate.

### INTRODUCTION

The monosaccharide constituents of serum glycoproteins are now known to be galactose, mannose, hexosamines (glucosamine and galactosamine), sialic

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acid and fucose, but fucose and galactosamine are minor constituents of some serum glycoproteins. Among them, sialic acid is coupled in glycosidic linkage to sugar, while in peptidic linkage to amino acid; therefore, sialic acid intermediated between sugar and amino acid. Pigman *et al.* (1964)<sup>1)</sup> and others<sup>2)3)</sup> investigated the combination of these substances. Also it is considered that sialic acid plays an important role in natural defense of the living body. There have been some interesting studies concerning the variations of serum and tissue sialic acid in patients with malignant neoplasm<sup>4)5)</sup>.

Serum glycoprotein levels have been detected to have a significant elevation in rheumatic disease, malignant neoplasm and inflammation in experimental animals as well as in humans<sup>6)7)</sup>. These high serum glycoprotein levels are now believed to be based on the increase of  $\alpha$  and  $\beta$  globulin, especially  $\alpha_2$  globulin. Clinically, it is very important to differentiate the diseases with high  $\alpha_2$  globulin and  $\gamma$  globulin. Consequently, these high glycoprotein levels are not specific in malignant neoplasm only.

The origin and formation of elevated serum glycoprotein levels have not been definitely attributed to malignant tumor. There are several concepts concerning sites of formation of serum glycoproteins; some believed that the high levels occurred during the processes involving tissue proliferation; Others concluded that the increased levels resulted from degradation of malignant neoplasm, and still others insisted that serum glycoproteins were produced in some organs representing reactions against unknown factors produced in malignant focus<sup>8)</sup>. But for the cancer researchers, considerable interest still exists in these problems<sup>9)10)</sup>. However, despite such investigations, little is known about the variation of serum glycoprotein in orthopaedic disease, and in particular no investigation has ever been done clinically about the changes of these serum components in malignant bone tumor. Furthermore, a review of the literature reveals no systematic research on these changes in the experimental animals with malignant bone tumor.

Nakagawa *et al.* (1966 and 1967)<sup>12)13)</sup> have suggested that urinary hydroxyproline excretion would be a good index representing turnover or breakdown in bone collagen, and that changes of serum hexosamine levels would be a significant index representing the variation in organic substrate of bone. It can not be said that the changes of serum glycoprotein levels reflect directly the lesions in organic substrate of bony tissues. The elevated values of serum glycoprotein, however, give a clue to what may be expected to happen in the patients with malignant bone tumor, *i.e.* osteogenic sarcoma and metastasis to bones, as similar changes were observed in the patients with malignant neoplasm of soft tissues. This study was undertaken as an initial step to define the variations of serum glycoprotein in the patients with malignant bone tumor. Changes of serum sialic acid and total hexosamine (glucosamine and galactosamine) levels, monosaccharide constituents of serum glycoprotein, were in-

vestigated in rats of the Sprague-Dawley strain as follows:

- (1) Physiological variations of these levels by age in normal rats.
- (2) Pathological variations of these levels in experimental rats.
  - a) These values during the growth of tumor after intramedullar implantation of Walker carcinosarcoma 256.
  - b) Relationship between these values and treatments with surgical operation, irradiation and anticarcinogen injection.
  - c) Changes of these values in corresponding to the recurrence of bone tumor and/or metastasis in the treated rats.

The isolated determinations of serum glucosamine and galactosamine, besides total serum hexosamine, were examined to find out the changes of galactosamine, which is found much more in bony tissues than glucosamine, where the specificity in malignant bone tumor is compared with malignant soft part neoplasm.

#### MATERIALS AND METHODS

##### *Materials*

The total number of 619 inbred Sprague-Dawley strain rats kept in the standardized environment were sacrificed in the present experiment. All rats were in the 2nd or 3rd delivery stage. The litter mates were artificially selected in number of eight after delivery and were weaned 3 weeks after birth. The rats were used for the experiment 4 weeks after birth. According to the standard body weight curve of the Sprague-Dawley strain rats in our raising room, this stage assumed to be the most suitable for the present experiment. All rats used for the experiment were fed with the regular solid food made by Asahi Electron Co., Ltd. and were given water freely during the experiment. The tumor of Walker carcinosarcoma 256, which was between the 12th and 17th generations of subcutaneous transfer, was used. About ten days after implantation, the subcutaneously transplanted tumor was removed aseptically and weighed. The peripheral parts of excised tumor, consisting of the tissue of histological sarcomatous pattern, were dissected into pieces the size of a rice or smaller. These small pieces were transplanted intramedullary into the left tibial condyle of the rat in the bone tumor group<sup>11)</sup>; and in the subcutaneously transplanted group, they were implanted subcutaneously at the midline of the back of the rats. In the sham operation group, only the hole drilling was done at the left tibial condyle of the rat at the same time. All rats were on antibiotic therapy for a day after operation. The surgical procedures were carried out under general anesthesia using intraperitoneal injection of Ouropan Soda (Shionogi Ph. Co., Ltd.) The rats were divided into seven groups as follows:

- (1) Normal control rats

The determination of serum sialic acid was made from 3 to 15 weeks of age in males, and from 3 to 10 weeks of age in females to eliminate the influence by pregnancy upon serum sialic acid levels. Total serum hexosamine (glucosamine and galactosamine) was estimated only in males between the age of 4 and 8 weeks.

(2) Bone tumor group and sham operation group

The rats with ulcerations on the surface of the induced bone tumor at 3 weeks after intramedullar implantations were excluded to eliminate the influence by the ulcerative inflammations upon serum sialic acid and total hexosamine levels. Sialic acid was measured on the sera of rats 2, 5, 7, 12, 14, 17, 21, and 28 days, and total hexosamine (glucosamine and galactosamine), 7, 14, and 21 days after intramedullar implantations respectively. In the sham operation group, serum sialic acid was evaluated on 1, 2, 3, 5, 7, 10, 14, 17, and 21 days, and total serum hexosamine (glucosamine and galactosamine), on 7, 14, and 21 days after sham operation.

(3) Subcutaneously transplanted group

Serum sialic acid was determined every other day and total hexosamine every four days after subcutaneous transplantation. All rats died within 18 days after subcutaneous implantation in this group.

(4) Remedied group

a) Amputated bone tumor group

The tumor-bearing leg was amputated at the upper one-third portion of the left femur after ligation of the femoral vessels on the 7th day after transplantation to confirm that the tumor became established. The suture was removed on the 5th day after operation and the rats whose operative wounds had been completely healed, were used for the determination. Serum sialic acid levels were estimated on the 7, 10, 14, 17, and 21 days after surgery, whereas total serum hexosamine (glucosamine and galactosamine) contents on the 7, 14, and 21 days after operation.

b) Irradiated bone tumor group

Rats of this group received irradiation of 500 R, in a single local exposure limited to the tumor-bearing legs to avoid irradiation damage to the other parts, one week after intramedullar implantation had been established. Serum sialic acid levels were measured 3, 7, 10, 14, 17, and 21 days after irradiation, and total serum hexosamine (glucosamine and galactosamine) contents, 3, 7, 14 and 21 days after irradiation.

c) Bone tumor group injected with cyclophosphamide

The rats whose tumor became established, were injected with cyclophosphamide (1,000  $\mu\text{g}/100\text{ g/day}$ : Endoxan: Shionogi Pharm. Co., Ltd.) intravenously in their tail vein 6, 7 and 8 days after the tumor transplantation. Serum sialic acid levels were determined on the sera of rats 3, 7, 10, 14, 17 and 21 days, and total hexosamine (glucosamine and galactosamine) contents, 7, 14

and 21 days after the 2nd injection (7 days after transplantation).

### *Methods*

#### (1) Determination of serum sialic acid

In 1959, Warren<sup>14)</sup> reported the thiobarbituric acid assay of tissue sialic acid applying the method for the measurement of 2-deoxyribose, which had been reported by Waravdekar and Saslow in 1957<sup>15)</sup>. In our country, Motegi *et al.* (1962)<sup>16)</sup> estimated serum sialic acid with this method and reported that the thiobarbituric acid assay was the most sensitive and specific for the determination of serum sialic acid levels. Therefore, the estimation of serum sialic acid levels was carried out by the thiobarbituric acid assay of Warren in the present investigation. N-acetylneuraminic acid, 90 per cent purified (U.S.A. Sigma), containing 5  $\mu$ g, 10  $\mu$ g and 20  $\mu$ g in 0.2 ml distilled water was used as standard, and distilled water as blank. The absorption spectra of N-acetylneuraminic acid (10  $\mu$ g) and one of sera were confirmed in each estimation of serum sialic acid levels and also interfering substances were confirmed to be of little influence. The recovery of the estimation was found between 92 and 98 per cent in this experiment, and a duplicate method was used in this assay.

#### (2) Determination of total serum hexosamine

Half ml of 6 N-HCl was added to 1 ml serum and the sample was hydrolysed in boiling water for fifteen hours. After cooling, the hydrolysate was filtered into a column of Dowex 50-X 8 to exclude interfering substances and the column was washed with 2 N-HCl and the elute was neutralized with 4 N-NaOH. The procedure for assay of total serum hexosamine using the neutralized elution was carried out with the modified Elson-Morgan reaction described by Boas (1953)<sup>17)</sup>.

#### (3) Determination of serum glucosamine and galactosamine

The developing colors due to glucosamine and galactosamine were in the same degree and the glucosamine and galactosamine could not be isolated individually in the procedure of Elson-Morgan reaction. The neutralized elution mentioned above, was used for assay of glucosamine and galactosamine with the method reported by Good *et al.* (1964)<sup>18)</sup>. The estimated values of glucosamine and galactosamine were simply shown as the ratio of glucosamine to galactosamine in this study.

## RESULTS

(1) Physiological variations of serum sialic acid and total hexosamine levels, and glucosamine/galactosamine ratio in the normal control rats.

As shown in Table 1, serum sialic acid levels indicated  $72.4 \pm 4.8$  mg/dl in male, and  $67.2 \pm 4.4$  mg/dl in female, 3 weeks of age. These levels had a tendency to gradual increase by age, and the changes of these levels in male

TABLE 1. Physiological Variations of Serum Sialic Acid and Hexosamine Contents, and Glucosamine/Galactosamine Ratio in Normal Rats of the Sprague-Dawley Strain Classified by Age

Age (week)	Male					Female		
	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.	Hexosamine (mg/dl) Mean±S.D.	Glucosamine/Galactosamine Mean±S.D.	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.
3	11	30.9±4.2	72.4±4.8			14	30.9±4.0	67.2±4.4
4	11	65.5±3.6	73.4±3.6	103.0±6.8	5.60±1.83	12	63.5±4.4	69.1±2.5
5	18	97.3±16.8	85.9±8.0	114.0±6.2	5.78±0.64	13	87.1±6.6	83.3±6.0
6	13	114.6±20.2	85.1±6.2	115.8±6.2	5.23±1.79	14	104.1±15.3	82.4±4.2
7	9	145.8±20.2	93.6±7.3	121.0±5.2	6.50±1.44	13	127.0±18.9	85.5±7.5
8	11	180.0±26.3	97.4±4.4	128.0±8.0	5.40±1.78	11	148.3±10.5	84.9±5.1
9	11	190.0±28.1	93.0±8.6			13	151.8±5.8	101.7±3.5
10	11	218.0±28.6	90.9±5.6			11	170.8±10.2	90.5±4.2
11	8	255.8±25.6	93.5±1.7					
12	7	278.3±13.3	97.0±3.9					
13	8	313.3±10.6	92.0±3.2					
14	8	338.5±22.8	96.7±0.9					
15	8	358.1±28.6	100.4±2.1					

In females, the estimation of sialic acid was made between 3 and 10 weeks of age to eliminate the influence by pregnancy upon serum glycoprotein levels. Both serum sialic acid and hexosamine contents were gradually increased by age. In the serum sialic acid values, no statistical significance was observed between males and females.

after 10 weeks of age, were also similar. The remarkable elevation during growth was not found. No statistical significance was observed between male and female in the serum sialic acid levels. Serum total hexosamine contents showed  $103.0\pm6.8$  mg/dl and  $128.0\pm8.0$  mg/dl 4 and 8 weeks of age in male rats respectively, and the variations of these contents by age were similar to those of serum sialic acid. The ratio of serum glucosamine to galactosamine was between 5.23 and 6.50, and showed no significant difference by age.

(2) Serum sialic acid and total hexosamine contents, and glucosamine/galactosamine ratio in the bone tumor and sham operation groups.

The size of tumor, that was intramedullarily implanted into the left tibia, was already as large as the tip of little finger on the 7th day after transplantation and became continuously larger which finally caused the death of the host within 3 or 4 weeks. This was a natural course of the progress of experimental bone tumor. The osteolytic and osteoplastic changes and spicula formation are well observed on the roentgenogram and these findings are similar to the osteogenic sarcoma in human (Photo. 1). The results observed are shown in Figs. 1, 2 and Table 2. Serum sialic acid level was already very high, *i.e.*  $113.0\pm4.2$  mg/dl on the 2nd day after transplantation, and continued to increase according to the growth of implanted bone tumor in this group. On the 4th week after transplantation, the value was  $160.7\pm1.9$  mg/dl. The remarkable increase would be caused not only by the growth of the implanted

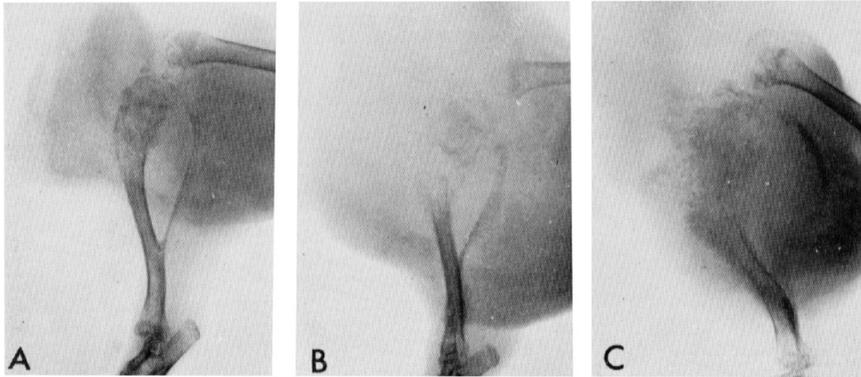


PHOTO 1. Roentgenograms of the left tibial condyle in male rats of the Sprague-Dawley strain, showing the growth of the transplanted Walker carcinoma 256. A, B and C each show the tumor development and the bone destruction in 1, 2 and 3 weeks after transplantation. The osteolytic and osteoplastic changes, and spicula formation are well observed, and these findings have a similarity to the osteogenic sarcoma in human.

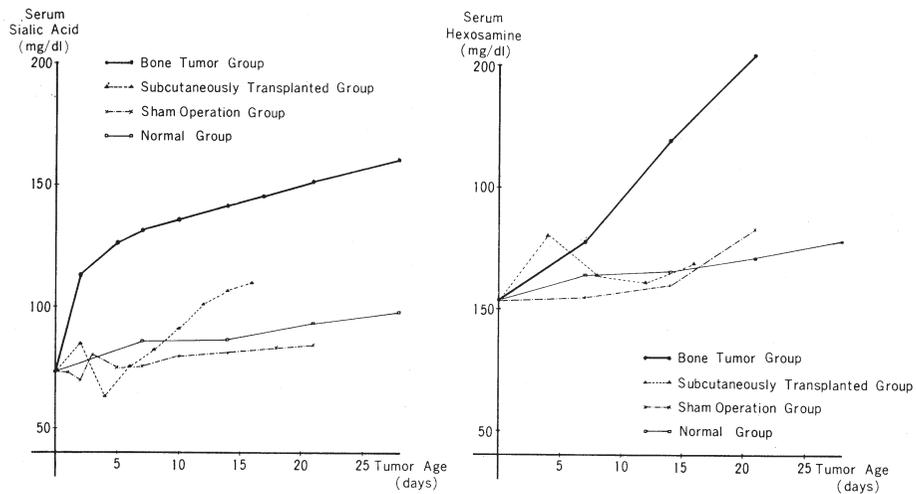


FIG. 1. Relationship between serum values of sialic acid (left) and hexosamine (right), and tumor age in the bone tumor group, subcutaneously transplanted group, sham operation group and normal group. The rate of successful transplantation in the left tibia was 90.4 per cent, but no failure was found in the subcutaneously transplanted group. The mean survival rate in rats subcutaneously transplanted was lower than that in rats intraosseously transplanted. Positive correlation coefficient existed between the wet weight of tumor and serum sialic acid levels in the subcutaneously transplanted group ( $r=0.3560$ ,  $p \leq 0.01$ ). There was a significant difference between the values in the bone tumor group and that in the subcutaneously transplanted group on the 14th tumor age (F-test,  $p \leq 0.05$ ). Also statistical significance between the value in the bone tumor group, and those in the normal and sham operation group existed respectively, in serum sialic acid levels in the 1st, 2nd and 3rd week, and in the hexosamine contents in the 2nd and 3rd week, after tumor transplantation (F-test,  $p \leq 0.05$  in hexosamine;  $p \leq 0.01$  in sialic acid).

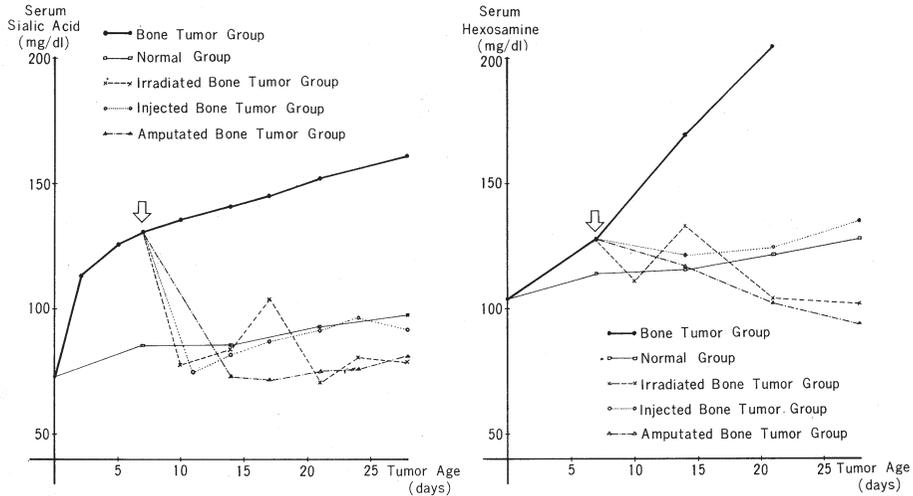


FIG. 2. Serum sialic acid (left) and hexosamine (right) values between normal and tumor transplanted groups, and the later consists of the groups of nontreated, irradiated and injected rats.  $\downarrow$  shows the time of amputation, irradiation (500 R) and cyclophosphamide (1,000  $\mu\text{g}/100 \text{ g/day}$ ; given respectively 6, 7 and 8 days after tumor transplantation). In each treated group, the number of recurrence and/or metastasis was 2 of 25 in the amputated bone tumor group, 8 of 40 in the irradiated bone tumor group and 8 of 33 in the injected bone tumor group. In each group, serum sialic acid levels rapidly decreased to normal values after treatment, but serum hexosamine contents fell slowly to normal range. There were statistical significances between the serum sialic acid levels of the bone tumor group and the other groups on the 14th, 21st and 28th tumor age (by the F-test  $p \leq 0.01$ ), and also serum hexosamine values on the 14th and 21st tumor age (by the F-test  $p \leq 0.05$  on the 14th tumor age and  $p \leq 0.01$  on the 21st tumor age).

tumor, but also by the ulcerations on the surface of neoplasm. Total serum hexosamine contents were similar to the tendency in serum sialic acid levels, and the content was  $204.2 \pm 9.1 \text{ mg/dl}$  on the 3rd week after transplantation. The ratio of serum glucosamine to galactosamine was increased according to the growth of implanted tumor. The alteration, however, was caused largely by the changes of serum glucosamine contents, and serum galactosamine levels varied slightly and slightly decreased in spite of the rising levels of total serum hexosamine in the amputated bone tumor group.

Fig. 1 and Table 3 show the variations of serum sialic acid and total hexosamine, and glucosamine/galactosamine ratio in the sham operation group. Serum sialic acid level was  $80.2 \pm 6.9 \text{ mg/dl}$  3 days after the sham operation, but, in general, the variations of serum levels of sialic acid and total hexosamine, and glucosamine/galactosamine ratio in this group had a similar tendency to those in the normal group, and no significant difference existed

TABLE 2. Serum Sialic Acid and Hexosamine Levels, and Glucosamine/Galactosamine Ratio Following the Tumor Age in the Group of Rats Transplanted the Walker Carcinosarcoma 256 into the Left Tibia

Tumor Age (days)	Male				
	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.	Hexosamine (mg/dl) Mean±S.D.	Glucosamine/Galactosamine Ratio Mean±S.D.
2	8	87.8± 5.3	113.0±4.2		
5	8	93.8± 9.1	126.0±5.4		
7	11	101.0± 5.3	131.0±7.5	127.2± 5.3	8.96±2.03
10	23	105.0±10.2	136.6±7.4		
14	20	128.0± 9.4	141.3±7.1	169.3±17.3	16.38±3.85
17	15	139.1±13.3	145.0±9.8		
21	18	147.0±13.9	152.3±9.2	204.2± 9.1	19.59±3.36
28	3	171.0± 7.5	160.7±1.7		

The value of glucosamine/galactosamine ratio was exceedingly increased following the growth of implanted bone tumor. The alteration was mostly caused by the changes of glucosamine contents, and the galactosamine levels had a small variation.

There was a statistical significance between the value in this group and that in the subcutaneously transplanted group on the 14th tumor age in the results of serum sialic acid estimation (by the F-test  $p \leq 0.05$ ).

TABLE 3. Serum Levels of Sialic Acid and Hexosamine, and Glucosamine/Galactosamine Ratio in the Sham Operation Group

Days after operation	Male				
	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.	Hexosamine (mg/dl) Mean±S.D.	Glucosamine/Galactosamine ratio Mean±S.D.
1	6	67.2± 6.7	73.1±4.1		
2	6	75.0±10.3	70.8±1.9		
3	6	70.5± 9.3	80.2±6.9		
5	7	85.4±10.3	75.7±5.1		
7	8	88.6± 8.1	75.4±4.3	105.2±7.8	5.06±0.78
10	7	101.4± 9.3	79.7±3.2		
14	8	112.2±10.4	81.1±1.4	110.4±2.7	6.53±0.98
17	8	139.2±15.3	83.3±3.7		
21	8	147.4±12.9	84.1±4.7	132.8±8.1	6.97±0.47

No statistical significance existed between the value in this group and in the normal group.

between the values in this group and normal group.

(3) Serum sialic acid and total hexosamine levels, and glucosamine/galactosamine ratio in the subcutaneously transplanted group.

In this group, serum sialic acid levels had a slight elevation on the 2nd day of tumor transplantation, but the lowest value with  $63.4 \pm 6.3$  mg/dl was observed 4 days after subcutaneous transplantation. The increasing tendency in serum values of sialic acid and total hexosamine in this group was not as

rapid as the changes in the bone tumor group, and there was a statistical difference between the serum values of both groups. As a result of decreasing serum galactosamine contents and increasing serum glucosamine levels, the ratio of serum glucosamine to serum galactosamine was found to be elevated in this group. There was a positive correlation coefficient between the wet weight of tumor masses subcutaneously transplanted and serum sialic acid levels ( $p \leq 0.01$ ,  $r = 0.3560$ , Fig. 1, Table 4).

TABLE 4. Serum Sialic Acid and Hexosamine Values, and Glucosamine/Galactosamine Ratio in the Subcutaneously Transplanted Group

Tumor age (days)	Male				
	No. of animals	Body weight (g) Mean $\pm$ S.D.	Sialic acid (mg/dl) Mean $\pm$ S.D.	Hexosamine (mg/dl) Mean $\pm$ S.D.	Glucosamine/Galactosamine ratio Mean $\pm$ S.D.
2	5	68.0 $\pm$ 6.4	85.2 $\pm$ 6.3		
4	5	65.0 $\pm$ 12.2	63.4 $\pm$ 6.3	130.4 $\pm$ 7.8	11.03 $\pm$ 1.34
6	5	74.6 $\pm$ 14.8	75.8 $\pm$ 7.0		
8	5	91.2 $\pm$ 14.0	82.2 $\pm$ 5.2	114.0 $\pm$ 5.3	5.22 $\pm$ 0.67
10	8	95.8 $\pm$ 9.7	92.3 $\pm$ 5.3		
12	7	128.3 $\pm$ 18.7	101.0 $\pm$ 6.0	111.5 $\pm$ 24.7	7.42 $\pm$ 0.10
14	9	109.6 $\pm$ 6.9	106.9 $\pm$ 11.2 <sup>†</sup>		
16	9	122.6 $\pm$ 14.2	110.1 $\pm$ 4.2	119.5 $\pm$ 24.2	9.63 $\pm$ 2.74

In comparison with the group of rats transplanted in the left tibia, not so remarkable were the increases of the values of serum sialic acid and hexosamine in this group. The glucosamine/galactosamine ratio increased following the growth of the subcutaneously transplanted tumor. The alteration was mostly caused by the changes of glucosamine contents, and the galactosamine levels had a small variation. The variation was similar to that of the bone tumor group, but the degree was not so remarkable as that of the bone tumor group.

<sup>†</sup> There was a significant difference between the value in this group and that in the bone tumor group on the same tumor age (by the F-test  $p \leq 0.05$ ).

(4) Serum sialic acid and total hexosamine values, and glucosamine/galactosamine ratio in the remedied bone tumor group.

a) Amputated bone tumor group

As shown in Fig. 2 and Table 5, serum levels of sialic acid returned to normal range with 73.4  $\pm$  6.4 mg/dl, 7 days after surgery and since then, the values have very slowly increased within physiological variations. There was a statistical significance between the values, on the 1st, 2nd and 3rd weeks after operation in this group and on the same corresponding weeks in the non-treated bone tumor group. On the other hand, serum total hexosamine contents also inclined to decrease in the course of 7, 14 and 21 days after amputation, but these contents returned under normal range 14 and 21 days

TABLE 5. Serum Sialic Acid and Hexosamine Levels, and Glucosamine/Galactosamine Ratio in the Group of Rats, Following Surgical Amputation of the Tumor-Bearing Leg at the Upper Portion of the Femur after Ligation of Femoral Vessels One Week After the Walker Carcinosarcoma 256 Implantation into the Left Tibia

Days after amputation	Male				
	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.	Hexosamine (mg/dl) Mean±S.D.	Glucosamine/Galactosamine ratio Mean±S.D.
7	5	115.4± 5.3	73.4±6.4*	117.6±7.8	11.47±0.23
10	5	121.2± 9.6	72.4±6.5		
14	5	150.6±13.5	75.2±2.9*	103.3±5.6*	5.67±0.28*
17	5	140.0±15.6	76.6±4.3		
21	5	156.7±25.3	81.2±4.0*	94.4±6.8*	5.03±1.73*

Serum sialic acid levels were rapidly fallen to the normal range, on the other hand, however, serum hexosamine contents were gradually decreased to the normal value in this group. The glucosamine/galactosamine ratio has become normal two weeks after surgical treatment. The rate of metastasis was 8 per cent. Serum sialic acid and hexosamine levels, and glucosamine/galactosamine ratio were indicated 117.0 mg/dl, 153.0 mg/dl, and 8.17 respectively, in one rat that was found the metastasis to lung by histological examination and to retroperitoneal lymph node. In another rat, having macroscopic metastasis to lung and retroperitoneal lymph node, serum sialic acid level was 137.0 mg/dl, serum hexosamine content 154.0 mg/dl and glucosamine/galactosamine ratio 7.49.

\* There was a significant difference between the value in this group and that in the bone tumor group (by the F-test  $p \leq 0.01$  in sialic acid,  $p \leq 0.05$  in hexosamine).

after treatments.

No statistical significance existed between the serum values of sialic acid and total hexosamine in the control rats whose left normal hind legs were amputated, and in the normal control rats on the 14th day after surgery. The ratio of serum glucosamine to galactosamine also fell within the normal limit after surgical treatment. The decrease was caused largely by the decrease of serum glucosamine contents. No recurrence of tumor at the stump occurred and no metastasis to the skeletal system was found by X-ray examination after amputation. Serum sialic acid and total hexosamine levels and glucosamine/galactosamine ratio indicated the high values of 117.0 mg/dl, 153.0 mg/dl and 8.17 respectively in one rat which had lung metastasis confirmed histological examination and metastatic focus as large as the tip of little finger in the retroperitoneal lymph node on the 21st day after surgical operation. Another rat had macroscopic tuberous metastasis in the lungs and a metastatic focus as large as an egg in the retroperitoneal lymph node at autopsy (Photo. 2), serum sialic acid level was 137.0 mg/dl, total serum hexosamine content 154.0 mg/dl, and glucosamine/galactosamine ratio 7.49. In both rats, the high ratios

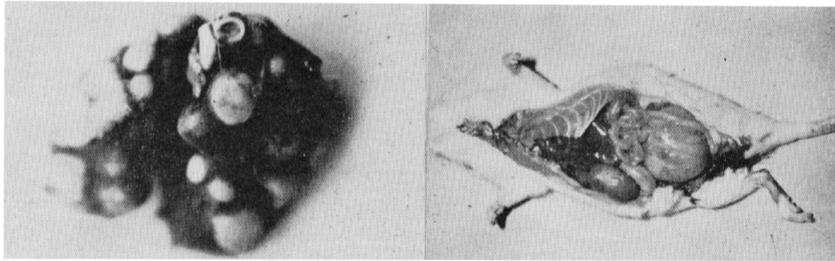


PHOTO 2. Shows a rat with lung (left) and retroperitoneal lymph node (right) metastasis 3 weeks after amputation of the tumor-bearing leg. Serum contents of sialic acid and hexosamine, and glucosamine/galactosamine ratio were high, *i.e.* 137 mg/dl, 154 mg/dl and 7.49 respectively in this rat. No metastasis to the skeletal system was found by X-ray examination in the same group.

of serum glucosamine to galactosamine were mainly caused by the elevation of serum glucosamine levels.

b) Irradiated bone tumor group

The data are summarized in Fig. 2 and Table 6. The ready response of the tumor to irradiation suggests that the implanted bone tumor had not increased in size and have begun to decrease in size about 4 to 5 days after the roentgenotherapy and then the lesions disappeared (Photo. 3). Serum

TABLE 6. Serum Values of Sialic Acid and Hexosamine, and Glucosamine/Galactosamine Ratio in the Group of Rats Whose Tumor-Bearing Legs were Exposed to Single Irradiation, 500 R, One Week After the Tumor Transplantation

Days after X-ray irradiation	Male				
	No. of animals	Body weight (g) Mean±S.D.	Sialic acid (mg/dl) Mean±S.D.	Hexosamine (mg/dl) Mean±S.D.	Glucosamine/Galactosamine ratio Mean±S.D.
3	6	85.6±7.3	78.3±4.7	111.33±4.2	9.52±1.96
7	5	119.0±29.1	85.8±5.8	133.4±12.4	8.34±2.01
10	5	114.0±13.7	104.4±5.5		
14	6	162.2±12.3	71.2±5.0	104.2±8.3	5.22±1.21
17	9	144.2±10.6	81.8±2.7		
21	9	134.8±21.7	79.8±6.7 <sup>△</sup>	102.0±18.4 <sup>△</sup>	5.32±1.56 <sup>△</sup>

The number of recidivation and/or metastasis was noticed 8 of 40 in this group. The levels of serum sialic acid, serum hexosamine and glucosamine/galactosamine ratio in 3 rats with recurrence and/or metastasis on the 3rd week after treatment are respectively as follows: 142.0 mg/dl, 180.0 mg/dl, 10.79; 104.0 mg/dl, 130.0 mg/dl, 7.63; 109.0 mg/dl, 151.0 mg/dl, 6.48.

<sup>△</sup> There was a significant difference between the value in this group and in the bone tumor group by the F-test ( $p \leq 0.01$  in sialic acid,  $p \leq 0.05$  in hexosamine).

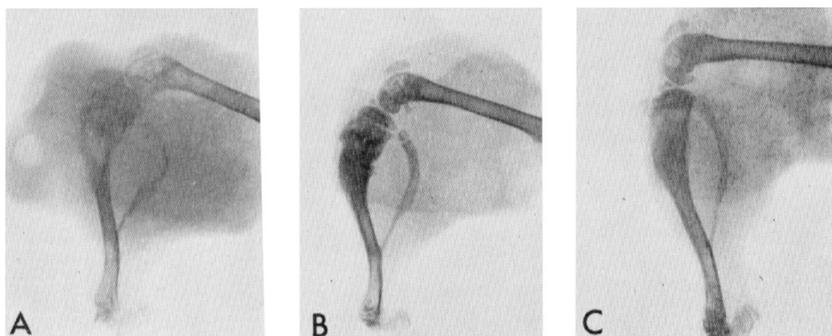


PHOTO 3. Roentgenograms of the tumor-bearing leg of the rat, exposed to single local irradiation of 500 R, showing reduction of tumor and remodeling of the destructive bone. The values of sialic acid and hexosamine, and glucosamine/galactosamine ratio were under normal range in the treated rat.

- A: 1 week after irradiation
- B: 2 weeks after irradiation
- C: 3 weeks after irradiation

sialic acid and total hexosamine levels returned to  $78.3 \pm 4.7$  mg/dl and  $113.3 \pm 4.2$  mg/dl respectively on the 3rd day after irradiation. The ratio of serum glucosamine to galactosamine, in parallel as total serum hexosamine levels, revealed slight elevation 7 day after X-ray therapy, but on the 21st day after radiation, returned to the normal value of  $5.32 \pm 1.56$ . The alteration was mainly due to the decrease of serum glucosamine levels as in the other treated groups. The number of recurrence and/or metastasis was noticed in 8 of 40 of this group. In the two of these rats, the tumor-bearing leg became slightly reduced after irradiation but since then, the implanted tumor had rapidly enlarged. In the rats with the local recurrence of tumor and/or metastasis, serum sialic acid levels were commonly elevated at a range of 102 to 142 mg/dl, and also in the total serum hexosamine and glucosamine/galactosamine ratio. There was no significant difference between the values of these components in this group on the 14th day after irradiation and in the control rats whose normal hind legs were exposed to the same dose of irradiation on the same corresponding day. Therefore, it might be considered that the influence on serum levels of these components by irradiation could be eliminated on the 14th day after irradiation.

#### c) Bone tumor group injected cyclophosphamide

There had been gradual decrease in size of the implanted bone tumor after intravenous injection of cyclophosphamide and the tumor disappeared, due to the effect of cyclophosphamide on the 21st day after the 2nd injection (Photo. 4). In rats with local recurrence of tumor, the recurred focus had increased in size about a week after the 2nd injection, and moreover, the rapid growth

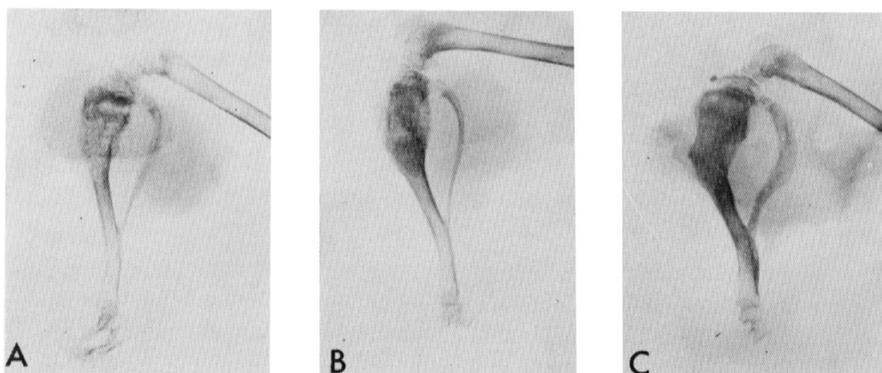


PHOTO 4. Roentgenographic changes in the tumor bearing leg of rats that were administered to intravenous injection of cyclophosphamide (1,000  $\mu\text{g}/100 \text{ g}/\text{day}$ ) 6, 7, and 8 days after tumor transplantation inclusively. The rate of recurrence and/or metastasis was high (about 24%) and the rate of healing was low in this group compared with other treated groups.

A: 1 week after 2nd injection of cyclophosphamide.

B: 2 weeks after 2nd injection of cyclophosphamide.

C: 3 weeks after 2nd injection of cyclophosphamide.

TABLE 7. Serum Levels of Sialic Acid and Hexosamine, and Glucosamine/Galactosamine Ratio in the Group of Rats Injected Intravenously of Cyclophosphamide (1,000  $\mu\text{g}/100 \text{ g}/\text{day}$ ), 6, 7 and 8 Days After the Tumor Transplantation

Days after the 2nd injection	Male				
	No. of animals	Body weight (g) Mean $\pm$ S.D.	Sialic acid (mg/dl) Mean $\pm$ S.D.	Hexosamine (mg/dl) Mean $\pm$ S.D.	Glucosamine/Galactosamine ratio Mean $\pm$ S.D.
3	5	103.8 $\pm$ 12.9	75.2 $\pm$ 3.7		
7	6	126.7 $\pm$ 20.3	83.6 $\pm$ 2.8	121.0 $\pm$ 12.05	9.64 $\pm$ 2.72
10	5	126.8 $\pm$ 17.2	88.2 $\pm$ 4.8		
14	6	133.5 $\pm$ 8.1	93.4 $\pm$ 4.3	124.5 $\pm$ 5.9	5.74 $\pm$ 0.43
17	5	131.5 $\pm$ 15.4	96.8 $\pm$ 5.7		
21	6	183.0 $\pm$ 13.3	92.0 $\pm$ 4.7 $\square$	135.25 $\pm$ 9.9 $\square$	8.63 $\pm$ 1.30 $\square$

Eight of 33 was the rate of recurrence and/or metastasis in this group. The values of serum sialic acid, serum hexosamine and glucosamine/galactosamine ratio in 3 rats with recurrence and/or metastasis 3 weeks after treatment respectively are as follows: 120.0 mg/dl, 141.0 mg/dl, 7.31; 131.0 mg/dl, 155.5 mg/dl, 9.97; 143.0 mg/dl, 193.0 mg/dl, 12.60.

$\square$  There was a significant difference between the value in the nontreated bone tumor group and in this group on the same tumor age (by the F-test  $p \leq 0.01$  in sialic acid,  $p \leq 0.05$  in hexosamine).

of the recurred bone tumor had been observed 14 days after the 2nd injection in comparison with that of the non-treated bone tumor. The results are included in Fig. 2 and Table 7. Serum sialic acid levels have gone down remarkable after cyclophosphamide injections, similar to the changes after operation in the amputated bone tumor group, *i.e.*  $75.2 \pm 3.7$  mg/dl by the 3rd day after the 2nd injection. Since then, the levels had been maintained within normal range. The decreasing of the serum levels of total hexosamine was also observed, but did not fall within the normal limit after injection of the anticarcinogen. The ratio of serum glucosamine to galactosamine that was caused by the declination of serum glucosamine contents, reached the normal values on the 21st day after the 2nd injection. Eight out of 33 was the rate of local recurrence and/or metastasis in this group. Serum levels of sialic acid and total hexosamine in the rats with local recurrence of tumor and/or metastasis were elevated with ranges of 104.0 to 145.0 mg/dl and 145.0 to 193.0 mg/dl respectively. On the 14th day after the 2nd injection, there was no statistical difference between the variations of serum sialic acid and total hexosamine levels in this group and those of the normal control rats that were given the same injection of cyclophosphamide.

(5) Relation between the serum levels of sialic acid and total hexosamine

There was a positive correlation coefficient between the values of serum sialic acid and total hexosamine in the groups of rats subcutaneously transplanted, intramedullarily transplanted, sham operated, and normal control rats ( $p \leq 0.01$ ,  $r = 0.9032$ ).

#### DISCUSSION

Many investigations upon the origin and significance of the changes in the serum glycoprotein in the malignant neoplasm have been reported by Shetlar *et al.* (1950) and other researchers<sup>19) 20)</sup>. The existence of the specific and qualitatively unusual glycoprotein in the specific disease has been supported by some researchers, while the appearance of these specific glycoprotein has been denied by other investigators. The relationship between the histological differences and the variations of serum glycoprotein levels should be of further study in the malignant tumor.

Since the elevations of the serum glycoprotein (as proteinbound hexose) level in rats bearing subcutaneous and intramuscular Walker carcinosarcome 256, were reported by Shetlar *et al.* in 1950<sup>19)</sup>, many studies have been represented on the variations of serum glycoprotein levels in malignant human tumor and in experimental neoplasm. In 1957, Weimer *et al.*<sup>21)</sup> demonstrated that the increased levels of serum glycoprotein were due to increase in 1 glycoprotein fraction, seromuroid. Machbeth *et al.* (1963)<sup>22)</sup> and Miettinen (1961)<sup>23)</sup> proved that there was a positive correlation coefficient between the variations of serum sialic acid levels and total serum hexosamine contents, and in this ex-

periment the same results were obtained ( $r=0.9032$   $p\leq 0.01$ ). But, on the contrary, Yamakawa (1957)<sup>24</sup> reported that the positive correlation coefficient did not always exist between the values of serum sialic acid and total hexosamine. Some effects of tumor implantation site on serum glycoprotein levels seem to be worthy of consideration as described by Kampshmidt *et al.* (1966)<sup>25</sup>.

In this study, the positive correlation coefficient existed between the wet weight of the extirpated tumor transplanted subcutaneously, and the serum sialic acid levels ( $r=0.3560$   $p\leq 0.01$ ). If the high glycoprotein levels were derived themselves directly from the glycoprotein in the neoplasm, the elevations of galactosamine would be expected in the serum being thought that the bone tumor had a specific genesis of bone tissue. But significant elevation of serum galactosamine levels had not been observed in rats with the Walker carcinosarcoma 256 transplanted subcutaneously or intramedullarily.

Molnar *et al.* (1965)<sup>26,27</sup> insisted that the neoplasm itself caused the elevation of serum glycoprotein levels, based on his experimental results obtained when intraperitoneal injection of <sup>14</sup>C-glucosamine were given to the mouse. As Shetlar (1961)<sup>8</sup> and Bekesi *et al.* (1966)<sup>28</sup> believed, however, the greater part of the elevated serum glycoprotein levels seemed to be derived from the promotion of serum glycoprotein synthesis, being caused directly or hormonally by an unknown factor that was produced in the proliferative tissues. Recently, the sialoprotein existing in the cell membrane is being studied in relation to the construction of cancer membrane, carcinogenesis, metastasis, infiltration and metabolism of energy in cancer cell. Further investigations would be able to clarify these problems.

The physiological variations of serum sialic acid levels by age in normal rats of the Sprague-Dawley strain were reported by Miettinen (1961)<sup>23</sup> and Dail *et al.* (1966)<sup>29</sup> in literature. These variations observed by them had a tendency to increase by age in comparison with present data, and these data could not be exactly compared with each because of different experimental conditions. On the other hand, however, the physiological variations of total serum hexosamine contents by age in rats of the Sprague-Dawley strain in this experiment are in accordance with the results described by Boas *et al.* (1953)<sup>30</sup> and Miettinen (1961)<sup>23</sup>.

The positive correlation coefficient existed between the levels of serum sialic acid and total hexosamine ( $r=0.9032$ ,  $p\leq 0.01$ ), and the ratio of serum glucosamine to galactosamine was within the constant range, although serum glucosamine and galactosamine levels that were estimated in rats between 4 to 8 weeks of age in this study, had rising tendency by age. The slight elevation of serum sialic acid level and total hexosamine content on the 3rd and 7th day after sham operation were observed in the rats of the sham operation group, but since then, both these values were maintained in the similar changes

to the levels in the normal control rats. In the bone tumor group, the level of serum sialic acid amounted to about 50 per cent of the levels in normal control rats 2 days after intramedullary transplantation, and the levels continued to elevate remarkably day by day until death 3 or 4 weeks after implantation. Total serum hexosamine contents elevated as high as those of serum sialic acid levels, and there was statistical significance between the levels of these values in this group and those in normal control rats ( $p \leq 0.01$ ). The changes of serum sialic acid and total hexosamine levels observed in intramedullary implantation rats were similar to those observed in intramuscularly injection of Walker carcinosarcoma 256, as described by Macbeth *et al.* (1963)<sup>22</sup>.

There was a statistical significance in the serum sialic acid levels between rats with bone tumor and with subcutaneous tumor on the 14th day after implantation, *i.e.*  $141.3 \pm 7.1$  mg/dl and  $106.9 \pm 11.2$  mg/dl, respectively, and also in the serum contents of total hexosamine. Then, judging from these data, it would be premature to explain that the lesions in the bone substrate lead directly to the alteration of these monosaccharide constituents in serum glycoprotein because the rats which had intramedullary implantation, had not only the lesions in bone substrate but also in the large neoplastic masses invading into the soft tissue. So special attention must be paid to the influence of these bone and soft part malignant foci on serum glycoprotein. It can not be denied, nevertheless, that the lesions in bone substrate reflect the changes of serum glycoprotein levels in bone tumor. In fact, the detailed investigation on the effects of tumor implantation sites on serum glycoprotein levels in bone tumor is a problem for further research.

Information of how the serum levels of sialic acid and total hexosamine change through treatments in patients with malignant bone tumor and in rats having experimental bone tumor, have not been obtained yet; besides, little investigation has been undertaken in the malignant soft part tumor in human and experimental animals<sup>31</sup>. The rats bearing the experimentally induced bone tumor, were treated with surgical amputation, irradiation and cyclophosphamide injection in this study, and consequently, the quick response to each treatment made the elevated levels of serum sialic acid and total hexosamine return to within normal range, but the processes of reaching the normal range rapidly occurred in serum sialic acid and gradually in total serum hexosamine.

In the determinations of serum glucosamine and galactosamine isolately, the following results were proved: when treatments cause total serum hexosamine levels to decrease within normal values, the decrease of glucosamine level was very remarkable in comparison with the variations of galactosamine in total serum hexosamine levels. The changes of serum glucosamine levels played an important role in the variation of total serum hexosamine, but the variations of serum galactosamine levels in each treated groups were not

similar because the decrease of serum galactosamine levels was observed in the amputated bone tumor group, and the slightly elevated level of serum galactosamine was detected in the irradiated bone tumor group.

The decreasing levels of serum sialic acid and total hexosamine through each therapy were again elevated following the recurrence of tumor and/or metastasis, but the degree of these elevation was not so remarkable compared with those in the bone tumor group. By deduction the positive correlation coefficients existed between the wet weight of the removed tumor which was subcutaneously transplanted and the serum sialic acid levels, and also between the values of serum sialic acid and total hexosamine. The levels of these constituents in serum glycoprotein may be concluded to be elevated in corresponding with the size of recurrence and/or metastatic focus. In recurrent and/or metastatic case, the elevated levels of total serum hexosamine were due to the increasing contents of serum glucosamine.

The surgical amputation was the most effective therapy, judging from the incidence of recurrence and/or metastasis, in which rats showed the elevations of these serum components in each treated bone tumor groups, and the doses of irradiation and anticarcinogen that were used in this study were less effective than those of surgery.

As a result of the experimental investigation, it may be considered useful clinically in following the response to therapy, the prognosis and for the early diagnosis of recurrence and/or metastatic recurrence, and to evaluate these components in serum glycoprotein in patients with malignant bone tumor. The variations of serum sialic acid and glucosamine levels after treatments in patients with stomach cancer were reported by Akita (1960)<sup>32)</sup> and Hano (1962)<sup>33)</sup>. So the findings of the experiment would be available to clinical practice in orthopaedic diseases, especially in the case of malignant bone tumor.

#### SUMMARY AND CONCLUSION

It is well known that serum glycoprotein levels have a tendency to increase by age and have a significant elevation in rheumatic disease, collagen disease, malignant neoplasm and inflammation in humans and in experimental animals. However, there have been no references concerning the elevation of serum glycoprotein in the host with malignant bone tumor. This investigation was carried out with the purpose of studying the variations of serum sialic acid and total hexosamine (glucosamine and galactosamine) levels, monosaccharide constituents of serum glycoprotein, in the host with malignant bone neoplasm. Sprague-Dawley strain rats were used in this experiment; namely, these variations were investigated to clarify the followings:

- 1) Physiological variations of these values due to age in normal rats.
- 2) Variations of these levels during the growth of tumor after intramedul-

lary implantations of Walker carcinosarcoma 256; relationship between these values and treatment; and changes of these values according to recurrence of tumor and/or metastasis in the treated rats.

As shown in the results of this study, the values of serum sialic acid and total hexosamine had a tendency to gradual increase according to age, and serum sialic acid and total hexosamine levels, and ratio of glucosamine to galactosamine showed significant increase with the growth of implanted tumor. Treatments of the tumor decreased these values to normal ranges, but these values were elevated again when the relapse of tumor and/or metastasis occurred. Positive correlation coefficients existed between the wet weight of tumor masses subcutaneously transplanted and serum sialic acid levels, and also between the levels of serum sialic acid and total hexosamine in this study. High contents of total hexosamine were largely caused by the changes of glucosamine levels and rarely by the changes of galactosamine levels.

Lesions of bony tissues did not increase galactosamine, which was found more in bone substrate than glucosamine, in the blood stream. So the following conclusions were obtained.

- 1) Serum sialic acid and total hexosamine are nonspecific reactive substances which are elevated in malignant neoplasm, inflammation, and collagen disease. Remarkable elevations of these values were also observed in the rats with the malignant bone tumor induced by Walker carcinosarcoma 256.

- 2) In the rats with intramedullary implanted bone tumor, treatments with surgical amputation, irradiation or anticarcinogen injection caused these values to normal ranges, these values, however, were again increased due to the recurrence of tumor and/or metastasis. Therefore, it may be considered valuable in observing the response to therapy and to estimate these values in prognosis.

- 3) Positive correlation coefficients existed between the wet weight of tumor masses subcutaneously transplanted and serum sialic acid levels, and also between the values of serum sialic acid and total hexosamine. As those results are taken into consideration, serum sialic acid and total hexosamine levels may be concluded to be elevated corresponding to the size of recurrent and/or metastatic focus.

- 4) The high contents of total hexosamine were chiefly caused by the changes of glucosamine levels and rarely by the changes of galactosamine levels in rats with intramedullarily implanted tumor.

- 5) Clinically it may be considered useful in observing the response to therapy, in prognosis and also for the early diagnosis of recurrence of tumor and/or metastasis, to estimate serum sialic acid and total hexosamine (glucosamine and galactosamine) levels in the patients with malignant bone tumor.

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