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CHROMOSOMAL PROTEINS CAPABLE OF INFLUENCING *IN VIVO* LIVER ENZYMES: ESPECIALLY, CATALASE AND PYRUVATE KINASE

by

Kaoru MIYAZAKI

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SUMMARY

Localization and characterization of *in vivo* liver catalase-depressing substance were studied, using rats.

1) Nuclei were isolated from Rhodamine sarcoma and from four different organs of normal rats; brain, spleen, kidney, and liver. When these nuclei were injected into mice, the catalase activities of the livers decreased in the order of "Rhodamine sarcoma">"brain">"kidney"="spleen">"liver". The homogenates of Rhodamine sarcoma and brain hardly showed catalase activity, whereas those of spleen and kidney showed approximately 10% and 40%, respectively, of the catalase activity of that of liver.

2) When nuclei from Rhodamine sarcoma were injected into mice three times every 24 hr, the catalase activities of the livers decreased to one-third of the original activity. By the injection of sarcoma nuclei into mice, the catalase activity with the soluble fraction from homogenates of the liver decreased more significantly than that with the particulate fraction from them. Immunological titration proved that the decrease of catalase activity in the livers of mice injected with sarcoma nuclei was brought about by decrease in the amount of catalase protein.

3) In the mice, whose liver catalase activity had been irreversibly inhibited by injection of 3-amino-1,2,4-triazole, the initial rate for the restoration of the liver catalase activity was significantly slowed by further injection of sarcoma nuclei. When the inhibitor

of catalase biosynthesis, allylisopropylacetamide, was injected into mice, the activity level of the liver catalase decreased. The extent of decrease by the injection of the inhibitor was slightly lower than that by the injection of sarcoma nuclei, which was almost the same as the extent of decrease by the injection of sarcoma nuclei plus allylisopropylacetamide.

It is conceivable that the catalase biosynthesis in the liver was inhibited by the injection of sarcoma nuclei in almost the same manner as by the injection of allylisopropylacetamide. However, it is not certain whether the degradation of liver catalase was slightly stimulated by the injection of sarcoma nuclei.

4) Pyruvate kinase in various tissues of rats was separable into seven kinds of pI-isozymes by isoelectric separation with Ampholine carrier ampholytes; pI 5.4-isozyme, pI 5.6-isozyme, pI 6.2-isozyme (two kinds), pI 6.6-isozyme, pI 7.4-isozyme and pI 7.8-isozyme. Some of these pI-isozymes contained bound fructose 1,6-diphosphate (FDP). The bound FDP was completely dissociated when the pI-isozymes were salted out with ammonium sulfate. In the FDP-free form, pyruvate kinase was classified into three types, liver-type (type L) of pI 6.2, muscle-type (type M) of pI 7.4 and spleen-type (type M_2) of pI 7.8. The liver- and spleen-type isoenzymes had two kinds of FDP-binding sites, but not at all the muscle-type isoenzyme.

When Rhodamine sarcoma grew on the back of rats, the content of spleen-type isoenzyme in the livers increased. When rats were injected with nuclei from Rhodamine sarcoma and with chromatin from the tumor

or spleen, the content of spleen-type isoenzyme in the livers again increased. This was not observed on the injection of chromatin from liver, indicating that the substance capable of increasing the spleen-type isoenzyme in liver was present in chromatins of sarcoma and spleen but barely or not at all in chromatin of liver.

5) Chromatin was prepared from Rhodamine sarcoma and then fractionated into the three components; histone, non-histone protein, and DNA. When these fractions were injected into mice, the catalase activities of the livers decreased with the histone and the non-histone protein but not with the DNA. At small doses, the extent of the decrease with the non-histone protein was significantly higher than that with the histone. Poly-L-lysine, a synthetic poly-cation, when injected into mice, also decreased the catalase activities of the livers to almost the same extent as the histone. By isoelectric separation, the non-histone protein was further divided into two fractions; one of pI <4 and the other of pI 4-7. When these two fractions were injected into mice, the pI 4-7 fraction decreased the catalase activities of the livers significantly at small doses, but not the pI <4 fraction.

These findings indicate that two different kinds of *in vivo* liver catalase-depressing substance are present in the chromatin; one is histone and the other a non-histone protein. Probably, the former is not different in kind and amount for all organs, and lower the catalase activity of a cell, the higher the content of *in vivo* liver catalase-depressing non-histone protein.

6) Nuclei prepared from Rhodamine sarcoma was heated in order to inactivate endogeneous protease and then washed with a mixture of chloroform and methanol (2:1), followed by extraction of chromatin. The chromatin thus obtained was fractionated into protein and DNA. When the protein fraction was further subjected to molecular-sieve fractionation on a Sephadex G-200 column and then to isoelectric separation, a single protein having a molecular weight of approximately 60,000 and a pI value of 5.0 was obtained. The purified acidic protein, when injected into mice, depressed the catalase activities of the livers to a significant extent. Therefore, it was concluded that the acidic protein was the original form of *in vivo* liver catalase-depressing substance.

INTRODUCTION

Greenstein and Andervont[1] reported that animals bear tumor, some enzymes in the livers are affected in activity. Of the several liver enzymes examined so far, the activity of catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is the most significantly decreased, regardless of the kind of tumor.[2,3] First of all, Nakahara and Fukuoka[4] demonstrated that when extracts from various kinds of tumor are injected into mice or rats, the catalase activities of the livers are decreased, designating the effective substance as "toxohormone". Since then, many workers attempted to purify and characterize toxohormone,[5-7] but their attempts have been mostly not successful; there are many discrepancies among their reports.[8,9]

Matuo *et al.*[10-12] found that in Rhodamine sarcoma, *in vivo* liver catalase-depressing substance (toxohormone) is bound with intracellular particles, and that it can be solubilized therefrom when the particles have been washed with a mixture of organic solvents. Using the soluble preparation, they succeeded in partial purification of the depressing substance. Recently, Kannan *et al.*[13] demonstrated that *in vivo* liver catalase-depressing substance is localized in the chromatin of Rhodamine sarcoma cells, and that in normal animals, it is present also in a nuclear fraction from dorsal muscle cells, but hardly in that from liver cells. On the other hand, Nakamura *et al.*[14] found that when Rhodamine sarcoma grows on the back of rats, or when chromatin prepared from the tumor is injected into rats, the content

of type M_2 isozyme of pyruvate kinase, which is the major isozyme in the tumor, significantly increases in the livers, indicating that the chromatin of Rhodamine sarcoma contains a substance capable of increasing type M_2 isozyme of pyruvate kinase in liver as well as *in vivo* liver catalase-depressing substance.

The present paper deals with studies on localization and characterization of *in vivo* liver catalase-depressing substance in chromatin of Rhodamine sarcoma.

MATERIALS AND METHODS

Animals and Tumor Adult male rats of the Donryu strain weighing 100-150 g and female white rabbits weighing approximately 2.5 Kg were purchased from Nihon Dobutsu Co., Ltd., Osaka. Male mice of the ddO strain weighing 15-20 g were supplied from the Junkei Dobutsu Jigyosha, Medical School, Osaka University. Animals were fed on a solid diet (Oriental Yeast Co., Ltd., Osaka). Rats bearing Rhodamine sarcoma (Umeda) were a gift from Professor S. Fujii, the Medical School, Tokushima University. The tumor tissue was hashed with scissors and then mixed with a small amount of streptomycin powder. Approximately 0.5 g in wet weight of the hashed tumor tissue was implanted into the subcutaneous region on the back of each rat. It took about 14 days for the planted tumor to grow into approximately 20% of the weight of the whole body.

Assay of Catalase Activity Catalase activity was assayed according to the method of Bonnischen *et al.*[15] The standard components of the reaction mixture for the assay of catalase activity were 0.4 ml of 30 m$\underline{\underline{M}}$ H_2O_2 and 3.2 ml of 0.1 $\underline{\underline{M}}$ sodium phosphate buffer (pH 7.0). A mixture of the standard components was incubated at 25° for 10 min. The reaction was started by adding 0.4 ml of an enzyme solution and stopped by adding 2.0 ml of 0.36 $\underline{\underline{M}}$ H_2SO_4 solution. Each of the reaction mixtures contained the amount of liver equivalent to 0.179 mg/ml in wet weight. With one enzyme solution, three reactions were carried out at 25° for 0, 20, and 40 sec. The amounts of H_2O_2 in the

resulting reaction mixtures were titrated with 2 m$\underline{\underline{M}}$ $KMnO_4$. Catalase activity is expressed by the value of K_{obs} (the first-order reaction rate constant) calculated by the following equation:

$$K_{obs} = \frac{2.3}{t} \log \frac{S_0}{S_t}$$

where S_0 and S_t represent the volumes of 2 m$\underline{\underline{M}}$ $KMnO_4$ required for titration of the reaction mixtures at the reaction time 0 and t sec, respectively. The average value of K_{obs} in the reactions for 20 and 40 sec is presented.

Assay of *in vivo* Liver Catalase-depressing Activity Assay of *in vivo* liver catalase-depressing activity was carried out by the method of Matuo *et al.*[12] Samples were dissolved in 0.15 $\underline{\underline{M}}$ NaCl or 0.01 $\underline{\underline{M}}$ sodium phosphate buffer (pH 7.0). For the assay, mice or rats were divided into some groups, one for the control and the others for the samples. The mice or rats for the sample groups were individually injected with 0.5-1.0 ml of a sample solution at the subcutaneous region on the back of the animals, and the mice or rats for the control with an equal volume of the same solvent as that used for dissolving the sample. The injected mice or rats were kept on water without supply of solid diet for 24 hr, except for the case of daily repeated injections of samples into mice or rats, in which the injected animals were kept with unlimited supply of water and diet. They were then decapitated and their livers were removed. The livers were separately homogenized in 7 volumes (v/w) of cold 0.1 $\underline{\underline{M}}$ sodium phosphate buffer (pH 7.0) with the use of a Potter-Elvehjem homogenizer.

Portions of the liver homogenates were futher diluted 70-fold with the buffer containing 0.3% Triton X-100. Each of the diluted solutions was immediately used as an enzyme solution for the assay of catalase activity described above.

Preparation of Nuclei and Chromatins from Various Tissues of Rats

Various tissues freshly obtained from rats were finely miced with scissors and then homogenized in 9 volumes (v/w) of 10 mM Tris-HCl buffer containing 0.25 M sucrose and 2 mM $MgCl_2$ (pH 7.5), with the use of a Potter-Elvehjem homogenizer. The resulting homogenate was filtered through 4 layers of gauze sheets in order to remove tissue debris. The filtrate thus obtained was centrifuged at 1,500 g for 10 min. For washing, the resulting precipitate was suspended in the same buffer solution as that described above, followed by centrifugation at 1,500 g for 10 min. The washed precipitate was suspended in 10 mM Tris-HCl buffer containing 0.25 M sucrose (pH 7.5). The resulting suspension was layered over 3 volumes (v/w) of 10 mM Tris-HCl buffer containing 1.7 M sucrose in centrifuge tubes, followed by centrifugation at 55,000 g for 3 hr. The precipitate thus obtained was washed twice with 7 volumes (v/w) of 0.15 M NaCl by centrifugation. The washed precipitate was suspended in the saline solution and used as the nucleus preparation. For the preparation of chromatin, the lastly washed precipitate was suspended in 5 volumes (v/w) of 0.2 mM EDTA (pH 7.0) and the suspension was homogenized for 3 min in a Waring blendor. The homogenized suspension was stirred at 4° for 2 hr in order to extract chromatin, and then centrifuged at 105,000 g for 30 min.

The supernatant was concentrated by ultrafiltration through an XM-100 membrane filter with the use of an Amicon Diaflo apparatus. The concentrated sample was used as the chromatin preparation. Approximately 50% of the amount of DNA present in the original tissue homogenate was recovered in the chromatin preparation.

Preparation of Histone from Chromatin of Rhodamine sarcoma Histone was prepared from chromatin according to the method of Fambrough *et al.*[16] The chromatin of Rhodamine sarcoma described above was dissolved in a volume of cold distilled water so that the DNA concentration of the resulting suspension would be lower than 500 μg/ml, followed by homogenization. The resulting solution, while being stirred in an ice-water bath, was slowly supplemented with one-fourth the volume of cold 0.5 *M* H_2SO_4. After further stirring for 30 min, the solution was centrifuged at 12,000 *g* for 30 min. The supernatant was saved, and the precipitate was resuspended in a smaller volume of cold 0.2 *M* H_2SO_4. The solution was stirred for 30 min and then centrifuged. These two supernatants were combined and supplemented with 4 volumes of cold ethanol in order to precipitate histone. When the mixture was stored below −10°, the precipitation of histone sulfate was completed within 24 hr. The mixture was then centrifuged at 12,000 *g* for 30 min. The white precipitate thus obtained was washed three times with cold ethanol, finely broken with a glass rod, and then dried in vacuum. The dried material was composed of basic protein (histone), free of acidic protein, DNA, or RNA.

Separation of Chromosomal Protein and DNA Present in Chromatin by Molecular-sieve Fractionation on Sephadex G-200 Column in High Concentration of Salt The chromosomal protein and DNA present in chromatin were separated by molecular-sieve fractionation on a Sephadex G-200 column, carried out in the presence of 2 M NaCl according to the method of Georgiev *et al.*[17] To 50 ml of chromatin solution containing approximately 150 mg of protein and 50 mg of DNA was added solid sodium chloride to make the final concentration to 2 M, followed by homogenization with the use of a Potter-Elvehjem homogenizer. The homogenate was vigorously stirred overnight in a cold room (4°) and then centrifuged at 105,000 *g* for 30 min. The clear supernatant was passed upward through a Sephadex G-200 column (5 x 100 cm) equipped with an upward-flow adaptor (Pharmacia Fine Chemicals, Uppsala). The charged column was developed with 2 M NaCl solution at a flow rate of 120 ml/hr controlled by a Mini Flow Micro-pump, type 4501 (LKB Produkter AB., Stockholm-Bromma). The eluate from the column was divided into 20-ml fractions. The DNA and protein contents of each fraction were estimated on the basis of absorbance at 260 nm and by the Lowry-Folin method, respectively.

Fractionation of Intracellular Particles of Mouse Liver Cells The livers removed from mice were perfused with an ice-cold 0.15 M NaCl solution to remove the blood, and the perfused livers were homogenized in 7 volumes (v/w) of 5 mM Tris-HCl buffer containing 0.25 M sucrose and 0.1% ethanol in order to prevent the formation of the inactive catalase complex II, with the use of a Potter-Elvehjem homogenizer.

The resulting homogenate (liver homogenate) was subjected to differential centrifugation according to the method of de Duve *et al.*[18] The cells and nuclei were precipitated by cectrifugation at 700 *g* for 10 min, the supernatant thus obtained was then centrifuged at 11,000 *g* for 15 min, and the resulting precipitate and supernatant were separated. The former was centrifugally washed with the buffer described above and the washed precipitate is called "particulate fraction". The supernatant was further centrifuged at 105,000 *g* for 1 hr and the supernatant thus obtained is called "soluble fraction".

Preparation of Catalase Antiserum Mouse liver catalase was purified by the method of Price *et al.*[19] Antiserum against catalase (catalase antiserum) was prepared according to the procedure of Higashi and Peters.[20] Ten mg of purified catalase showing a specific activity of 3.7×10^7 $M^{-1}sec^{-1}$ was emulsified in an equal volume of complete Freund's adjuvant (Difco), and injected intramuscularly into female white rabbit weighing 2.5 Kg. Four weeks after the injection, a booster injection of 4 mg catalase was given. One week after the booster injection, the rabbit was bled, and the serum was collected by severing the carotid artery and stored at -20°. This serum is called "catalase antiserum" and it was positive in the ring test at 1:64 dilution.

Quantitative Analysis of Catalase Protein The amount of catalase protein present in liver extracts was estimated by the immumotitration method of Ganschow and Schimke.[21] Mouse liver was homogenized in 10 volumes (v/w) of 50 m*M* sodium phosphate buffer (pH 7.0). To the

resulting homogenate, one volume (v/v) of 1.0% sodium deoxycholate solution was added in order to solubilize the particle-bound catalase. The resulting solution was incubated at 0° for 30 min, followed by centrifugation at 105,000 g for 60 min. The clear supernatant thus obtained (deoxycholate liver extract) was subjected to quantitative analysis of catalase protein. Catalase antiserum was added to a definite volume of the deoxycholate liver extract, and the mixture was incubated at 37° for 30 min and then at 4° for 24 hr. During this incubation, immunoprecipitate was formed and removed by centrifugation. The catalase activity remaining in the resulting clear supernatant was determined. The amount of catalase antiserum to be added to the deoxycholate liver extract was varied. The values of catalase activity thus measured were plotted as a function of the amount of catalase antiserum (see Fig. 4). The minimum amount of catalase antiserum required to precipitate all the catalase present in the deoxycholate extract was estimated by extrapolating the linear portion of the immunotitration curve.

Assay of Pyruvate Kinase Activity The activity of pyruvate kinase was measured according to the method of Bücher and Pfeiderer.[22] The standard reaction mixture comprized 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.5), 0.1 ml of 20 mM phosphoenolpyruvate, 0.1 ml of 20 mM ADP, 0.1 ml of 1.5 M KCl, 50 µl of 0.2 M MgCl, 50 µl of 5 mM NADH, 10 µl of 150-200 units/ml of lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), and water to make the total volume to 1.0 ml. In some cases, fructose 1,6-diphosphate was added to the

standard reaction mixture. The reaction was started by adding 10 or 20 μl of enzyme sample to the standard reaction mixture, and the decrease of absorbance at 340 nm was measured at 24°, using a Cary model 17 spectrophotometer or a reaction rate analyzer (LKB-Produkter AB., Stockholm-Bromma). One unit of pyruvate kinase was defined as the amount of enzyme that caused the oxidation of 1 μmole of NADH *per* min.

Analysis of Pyruvate Kinase pI-Isozymes in Various Tissues of Rats

Normal and tumor-bearing rats were decapitated, and various organs and the tumor were dissected out. Extracts from various tissues were prepared by the procedure of Nakamura *et al.*[14] The extracts, without desalting, were subjected to isoelectric separation with Ampholine carrier ampholytes according to the method described below. In some cases, solid ammonium sulfate was added to the above extracts to 70% saturation, followed by centrifugation at 20,000 *g* for 20 min. The resulting precipitates were individually dissolved in 0.1 *M* Tris-HCl buffer (pH 7.5) containing 5 m*M* ethylenediamine tetraacetate (EDTA) and 10 m*M* 2-mercaptoethanol. The solution thus obtained were desalted on a Sephadex G-25 column. This process is called "ammonium sulfate treatment". The resulting desalted solutions were subjected to isoelectric separation. After electrolysis for 40 hr at 0-2°, the eluate from the column was fractionated and the resulting fractions (1 ml) were measured for pH, absorbance at 280 nm and pyruvate kinase activity.

Isoelectric Separation Isoelectric separation was carried out with

the use of Ampholine carrier ampholytes according to the method of Vesterberg and Svensson.[23] Ampholine carrier ampholytes were purchased from LKB-Produkter AB., Stockholm-Bromma. A solution of the ampholytes capable of producing a gradient of pH from 3 to 10 was used at a final concentration of 1% (w/v). The electrolysis was carried out at 0-2° in a 100-ml column, which was designed by us. After electrolysis for approximately 40 hr (approximately 700 V and 1.3 mA at the steady state), the content was eluted from the bottom of the column and divided into 1- or 2-ml fractions. The resulting fractions were measured for pH at 0-2° and then other properties, such as absorbance at 280 nm and enzyme activity.

Determination of Contents of Protein, DNA and RNA The content of protein was determined by the method of Lowry *et al.*[24] with the use of human serum albumin as the standard. The content of DNA was determined by the diphenylamine reaction of Schneider,[25] and the content of RNA by the orcinol reaction, with the use of herring sperm DNA and yeast RNA as the standards, respectively.

Reagents Allylisopropylacetamide was a gift from the Hoffman-La Roche & Co., Switzerland. 3-Amino-1,2,4-triazol was purchased from Nakarai Chemicals, Ltd., Kyoto. Poly-L-lysine was purchased from Protein Research Foundation, Osaka. ADP was purchased from Sigma Chemicals Co., St.Louis, Missouri. Sodium phosphoenolpyruvate and fructose 1,6-diphosphate were purchased from Boeringer Manheim GmbH, Manheim. NADH and lactate dehydrogenase were obtained from Oriental Yeast Co., Ltd., Osaka.

RESULTS

I. Content of *in vivo* Liver Catalase-depressing Substance in Nuclei Isolated from Various Tissues of Rats

I-1. *In vivo* Liver Catalase-depressing Activities of Nuclei from Vaious Tissues of Rats

It was previously reported by Kannan *et al.*[13] that the nuclear fractions from Rhodamine sarcoma and from dorsal muscles of normal rats, but hardly the nuclear fraction from livers of normal rats, depressed the activity of liver catalase to a significant extent when the fractions were injected into mice. In the present study, nuclei were prepared from brains, spleens, kidneys and livers of normal rats, as well as Rhodamine sarcoma, and the *in vivo* liver catalase-depressing activities of these nuclei were compared.

Nuclei were prepared from Rhodamine sarcoma of rats and the four different kinds of tissues of normal rats; brain, spleen, kidney, and liver. The contents of protein, DNA, and RNA of the nucleus preparations were determined (Table I). The ratio in weight of the

Table I.

protein to the DNA varied from 2 to 4. These preparations were stored in a frozen state without loss of *in vivo* liver catalase-depressing activity. Before use, they were melted and homogenized. An assumption was made that cells individually contained the same

amount of DNA in the nuclei, regardless of the kind of tissue of rats. On the basis of this assumption, nucleus preparations from different kinds of tissues were injected into mice weighing 20-25 g at three different doses, 0.45, 1.8, and 4.5 mg, in DNA (Table II).

Table II.

Therefore, the protein contents at each of these doses were somewhat different in nucleus preparations from different kinds of tissues. For example, at a dose of 0.45 mg in DNA, the nucleus preparations from Rhodamine sarcoma and from brain contained 1.8 and 1.5 mg of protein, respectively. The Rhodamine sarcoma nuclei showed the highest depressing activity, indicating that it contained the highest amount of *in vivo* liver catalase-depressing substance. On the contrary, the liver nuclei hardly showed the depressing activity. These results are in good accordance with those obtained by Kannan *et al*.[13] The brain nuclei showed the depressing activity to a significant extent, although the extent was somewhat lower than that with the sarcoma nuclei. The depressing activities with the kidney and spleen nuclei were lower than that with the brain nuclei.

I-2. Catalase Activities of Various Tissues of Rats The catalase activities of the homogenates of Rhodamine sarcoma, brain, spleen, kidney, and liver were measured (Table III). The liver homogenate

Table III.

showed the highest catalase activity, whereas the sarcoma and brain homogenates hardly showed catalase activity. The catalase activities of the kidney and spleen homogenates were approximately 40% and 10% of that of the liver homogenate, respectively.

On a comparison between the catalase-depressing activities of the nuclei and the catalase activities of the tissues from which the nuclei were obtained, it may be accepted that the content of the catalase-depressing susbstance in nuclei is nearly in inverse proportion to the catalase activity level of the original tissue whether the tissue is normal or malignant.

II. Effect of Daily Repeated Injections into Mice of Nuclei from Rhodamine sarcoma on Liver Catalase

II-1. Change of Catalase Activity in Mouse Liver by Daily Repeated Injections of Nuclei from Rhodamine sarcoma and Liver As described previously, it was found that the nucleus preparation from Rhodamine sarcoma showed the highest liver catalase-depressing activity of the examined five nucleus preparations, but the liver nucleus preparation did the lowest one. In the present study, both nucleus preparations were daily injected into mice three times, and the effect of the injections on the liver catalase activity was examined.

Normal mice (approximately 30 g in body weight) were divided into four groups. Each group consisted of 3-4 mice. One group was injected with 0.15 M NaCl solution and the remaining three groups were injected with 4.8 mg in protein of the sarcoma nucleus preparation. Of these three groups, two groups were again injected with

4.8 mg of the sarcoma nucleus preparation 24 hr after the first injection. Of the resulting two groups, one group was further injected with 4.8 mg of the sarcoma nucleus preparation 24 hr after the second injection. Injections were made subcutaneously on the back of mice. All the mice were decapitated 24 hr after the respective last injection, and the catalase activity in the liver was determined (Fig. 1).

Fig. 1

In the group given one injection of the sarcoma nucleus preparation, liver catalase activity was depressed to approximately 60% of the original activity 24 hr after the injection. Doses higher than 4.8 mg hardly increased the extent of depression. Unless mice were further injected with the sarcoma nucleus preparation, the depressed activity was restored at such a slow rate that it would reach almost the original level approximately 3 days after the injection. On the other hand, injection of the sarcoma nucleus preparation two and three times caused further depression of liver catalase activity. When the injection was made three times, liver catalase activity was depressed to approximately 20% of the original activity. In repeated experiments, the extent of depression by three times of injection varied from 60% to 85%.

When the liver nucleus preparation was injected into mice in the same manner as the sarcoma nucleus preparation, catalase activities

of the livers were depressed to some extent (Fig. 2). However, the

Fig. 2

extent of depression was significantly lower than that with the sarcoma nucleus preparation; more than two-thirds of the original activity remained 24 hr after the third injection.

II-2. Effect of Injection into Mice of Nuclei from Rhodamine sarcoma on Catalase Activities of Particulate and Soluble Fractions from Livers Normal mice were injected with the sarcoma nucleus preparation in the same manner as that described above. Liver cells from the injected mice were disrupted and fractionated into particulate and soluble fractions. The resulting two fractions were then subjected to the assay of catalase activity (Fig. 3). It was

Fig. 3

found that, by the injection of the sarcoma nucleus preparation, the catalase activity in the soluble fraction decreased more rapidly than that in the particulate fraction. By injection three times, the activities in the soluble and particulate fractions were depressed to approximately 25% and 50% of the original activity, respectively. The extent of depression varied somewhat from one experiment to another. These findings are in agreement with Price and Greenfield,[26] who found that in the liver of tumor-bearing animals, catalase activity

in the soluble form is more significantly depressed than that in the particle-bound form.

II-3. Effect of Injection into Mice of Nuclei from Rhodamine sarcoma on Amount of Catalase Protein in Livers The livers of mice with and without one injection of the sarcoma nucleus preparation were extracted with 0.5% sodium deoxycholate (pH 7). It was found that all the catalase in the particle-bound form was solubilized in the presence of the detergent. Immunotitration was carried out with the resulting liver extracts; the liver extracts from mice with and without injection of the sarcoma nucleus preparation showed K_{obs} values of 1.1×10^{-2} and 2.4×10^{-2}, respectively. The minimum amount of catalase antiserum required for precipitation of all the catalase present in 0.4 ml of the liver extract (5 mg% in wet weight) was determined by extrapolation of the linear portion of the immunotitration curve. Doubtless, the minimum amount thus obtained corresponds to the relative amount of catalase present. The volumes of catalase antiserum were 0.11 ml and 0.2 ml, respectively, for 0.4 ml of the liver extracts from the mice with and without one injection of the sarcoma nucleus preparation (Fig. 4). This

Fig. 4

proved that when the sarcoma nucleus preparation was injected, the amount of liver catalase decreased. In the livers of mice injected with the sarcoma nucleus preparation three times every 24 hr, the decrease in catalase activity was in parallel to the decrease in

catalase protein (Fig. 5). This finding is comparable to findings

Fig. 5

by other laboratories that the depression of liver catalase activity either in tumor-bearing animals or in the animals injected with the toxohormone is accompanied by decrease in the amount of catalase protein.[27-29]

III. Effect of Injection into Mice of Nuclei from Rhodamine sarcoma on Turnover of Liver Catalase

III-1. Effect of Injection of Nuclei from Rhodamine sarcoma on Rate for Restoration of Catalase Activity after Injection of 3-Amino-1,2,4-triazole It is well known that the activity of catalase is irreversibly inhibited by 3-amino-1,2,4-triazole (aminotriazole). Intraperitoneal injection of the inhibitor into animals causes a rapid and significant decrease in activity of the catalase present in the liver, and this decreased catalase activity is gradually restored to the original level. Price *et al.*[19] studied the rate for the recovery of catalase activity in rat liver after injection of aminotriazole, and verified that the synthesis of new catalase protein is responsible for the recovery. We repeated their experiment in order to examine the effect of injection of the sarcoma nuclelus preparation into mice on the turnover of catalase in their liver. The liver catalase activity was almost completely inhibited 2 hr after the injection of 30 mg of aminotriazole into mice weighing

approximately 30 g, and restored to approximately 85% of the original level after 3 days (Fig. 6). When 4.8 mg in protein content of the

Fig. 6

sarcoma nucleus preparation was injected into mice three times every 24 hr, in addition to the single injection of aminotriazole at time 0, the restoration was significantly lowered both in rate and extent. This indicates that the injection of sarcoma nucleus preparation caused an impaired turnover (the diminished rate of synthesis and/or the enhanced rate of degradation) of catalase in the liver, in accordance with the findings with the toxohormone.[29,30)]

Liver extracts were divided into particulate and soluble fractions according to the method described above. It was found that, in the mice injected with aminotriazol, the catalase activity in the particulate fraction was restored at a significantely faster rate than that in the soluble fraction (Fig. 7). Three days after

Fig. 7

the injection of aminotriazol, the catalase activity in the particulate fraction was restored almost completely, while the catalase activity in the soluble fraction was restored to one-half of the original level. This suggests that the turnover rates of liver catalase were significantly different between the particle-

bound and the free forms. When the sarcoma nucleus preparation was injected three times every 24 hr, the restorations of catalase activity in the particulate and soluble fractions were depressed both in rate and extent. Three days after the first injection of the sarcoma nucleus preparation, the catalase activities in the particulate and soluble fractions were 40% and 25% of the original levels, respectively.

III-2. Change of Catalase Level in Liver by Injection of Allylisopropyl-acetamide with and without Nuclei from Rhodamine sarcoma Schmid *et al.*[31] reported that allylisopropylacetamide inhibits the biosynthesis of catalase in the liver. In the present study, the reagent (2 mg/10 g body weight of mouse) was injected into mice three times every 24 hr to inhibit the biosynthesis of catalase. The catalase activity level in the liver decreased gradually by injections of allylisopropylacetamide; it was 35% of the original level three days after the first injection (Fig. 8). This

Fig. 8

suggests that when catalase biosynthesis was inhibited, the catalase activity level in the liver lowered by *in vivo* degradation of the enzyme. When the sarcoma nucleus preparation was injected together with allylisopropylacetamide, the *in vivo* degradation of catalase was slightly faster than when allylisopropylacetamide alone was injected. However, it was found that the injection of the sarcoma

nucleus preparation alone gave nearly the same degradation rate as the injection of the sarcoma nucleus preparation + allylisopropylacetamide. If the sarcoma nucleus preparation stimulated the catalase degradation but did not inhibit catalase biosynthesis, the apparent rate of catalase degradation ("degradation rate" minus "biosynthesis rate") would be remarkably faster in the case of injecting the sarcoma nucleus preparation plus allylisopropylacetamide (inhibitor of biosynthesis) than in the case of injecting the sarcoma nucleus preparation alone. This was not the case. Therefore, it is conceivable that the catalase biosynthesis in the liver was inhibited by the sarcoma nucleus preparation in almost the same manner as by allylisopropylacetamide. The fact that the sarcoma nucleus preparation invoked slightly faster rate for the decrease of catalase activity level than allylisopropylacetamide suggests the possibility that the sarcoma nucleus preparation slightly stimulated catalase degradation in the liver in addition to the inhibition of catalase biosynthesis. However, further experiment is needed before such a conclusion can be reached because it is not certain at present whether the inhibition of catalase biosynthesis by allylisopropylacetamide was the same in extent as that by the sarcoma nucleus preparation.

IV. Effect of Injection into Rats of Nuclei or Chromatin from Rhodamine sarcoma on Pyruvate Kinase Isozymes in Livers

In 1965, Tanaka and his associates[32] observed two types of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) in rat liver; these were separable electrophoretically. In 1967, they[33] crystallized the major type of the liver enzyme (type L) and a single

type of the muscle enzyme (type M). These two types are distinguishable electrophoretically, immunologically, kinetically, in reactivity to p-chloromercuribenzoate, in molecular weight, etc. Since then, there have been many publications describing multiple forms of pyruvate kinase in various tissues.[34] Bailey et al.[35] found that type L, but not type M, is allosterically activated by fructose 1,6-diphosphate.

Suda et al.[36] reported that type M pyruvate kinase content increased in the livers of Walker sarcoma-bearing rats.

Tanaka et al., [33] using a block zonal electrophoresis, found four isozymes of pyruvate kinase from rat liver; one of these four isozymes was identical with type M isozyme as regards electrophoretic mobility. However, Susor and Rutter[37] claimed, using electrophoresis on a cellulose acetate membrane, that the type M isozyme present in the liver was different from the isozyme present in the muscle. This conclusion was supported by Jiménez De Asúa et al.[38]

Imamura and Tanaka[39] have succeeded in the purification of a new type of pyruvate kinase from Ehrlich ascites tumor of rats, designating it "type M_2". Nakamura et al.,[14] by isoelectric separation with Ampholine carrier ampholytes, demonstrated that pyruvate kinase present in various tissues of the rat is separable into five pI-isozymes (differing in pI value), and observed that type M_2 (type K) was the major isozyme in kidney as well as in Rhodamine sarcoma, and that it was also present in liver, spleen, lung and

erythrocytes. In addition, they demonstrated that type M_2 content increased in the livers of rats injected with chromatin prepared from the nuclei of tumor cells. Recently, Ibsen, Basabe and Lopez[40] carried out similar experiments using Ehrlich ascites tumor cells, and their findings were in good accord with those of Nakamura *et al.*[14]

In the present study, the relationships among the various pI-isozymes present in various tissues of rat were studied, together with the effect of injection into rats of nuclei or chromatins from Rhodamine sarcoma on pyruvate kinase isozymes in the livers.

IV-1. Isoelectric Separation of pI-Isozymes of Pyruvate Kinase Present in Various Tissues of Normal Rats

1-a) Liver: When extracts from livers of normal rats were subjected to isoelectric separation, the pyruvate kinase was separable into pI 5.4-, pI 5.6-, pI 6.2-, pI 7.4- and pI 7.8-isozymes (Fig. 9). The pI 5.4-isozyme thus separated, if subjected again to

Fig. 9

isoelectric separation, was mostly converted into the pI 5.6- and pI 6.2-isozymes (Fig. 10). In repeated isoelectric separation, pI 5.6-

Fig. 10

isozyme was converted into the pI 6.2-isozyme, whereas the pI 6.2-

isozyme did not change. When liver extracts were subjected to ammonium sulfate treatment (as described previously) and then isoelectric separation, pI 5.4- and pI 5.6-isozymes were hardly detectable, whereas the pI 6.2-isozyme content increased significantly and pI 4.9-isozyme was newly formed; pI 7.4- and pI 7.8-isozymes were hardly affected (Fig. 9). The resulting pI 4.9-isozyme was labile, and it was not examined further in the present study; it seems likely that this isozyme was an artifact generated by the ammonium sulfate treatment. The resulting pI 6.2-isozyme was not influenced by repeated ammonium sulfate treatment.

Hess *et al.*[41] found, using the isoelectric separation method, that two pI-isozymes of pyruvate kinase are present in pig liver extracts, and that one is convertible into the other by binding with fructose 1,6-diphosphate. Therefore, it is conceivable that the conversion of pI-isozymes by repeated isoelectric separation or ammonium sulfate treatment resulted from partial or complete dissociation of fructose 1,6-diphosphate from enzyme molecules. In addition, ammonium sulfate treatment seems more effective than repeated isoelectric separation in the dissociation of fructose 1,6-diphosphate.

In the present study, the liver pI 6.2-isozyme fractions obtained by isoelectric separations before and after ammonium sulfate treatment were mixed with an equal volume of 0.2 m*M* fructose 1,6-diphosphate, and the resulting mixtures were again subjected to isoelectric separation. It was found that the pI 6.2-isozyme was

converted into pI 5.4- and pI 5.6-isozymes and the ratio of activity of the former to the latter was approximately 2:1. This ratio increased with increasing concentrations of fructose 1,6-diphosphate. The resulting pI 5.4- and pI 5.6-isozymes were convertible into pI 6.2-isozyme by the procedure described above. However, if the isoelectric separation was prolonged, the pI 5.4-isozyme initially formed was gradually converted into pI 5.6-isozyme and then into pI 6.2-isozyme.

These results indicate that the three pI-isozymes present in liver extracts are identical in protein species (liver-type isoenzyme), and that this isoenzyme probably possesses two kinds of sites capable of binding with fructose 1,6-diphosphate; pI 5.6- and pI 5.4-isoenzymes are types bound with fructose 1,6-diphosphate at one and two kinds of sites, respectively, and pI 6.2-isozyme is a type free of the sugar phosphate.

1-b) Spleen: When extracts from spleens of normal rats were subjected to isoelectric separation, the pyruvate kinase was divided into pI 6.2, pI 6.6-, pI 7.4- and pI 7.8-isozymes (Fig. 11). In this

Fig. 11

experiment, pI 6.2-isozyme content was significantly higher than pI 6.6-isozyme content. However, in some spleen extracts, the pI 6.6-isozyme content was significantly higher than that of pI 6.2-isozyme, the total amount of these two isozymes being similar in all spleen extracts. The resulting pI 6.2-isozyme, if it was again

subjected to isoelectric separation, was mostly converted into pI 6.6-isozyme, indicating that this pI 6.2-isozyme was obviously different from the liver pI 6.2-isozyme in spite of the similar pI value (Fig. 12). The pI 6.6-isozyme was not affected by repeated

Fig. 12

isoelectric separations. When spleen extracts were subjected to ammonium sulfate treatment and then to isoelectric separation, the pI 6.2- and pI 6.6-isozymes were not observable, whereas the pI 7.8-isozyme content increased significantly (Fig. 11). The pI 7.8-isozyme obtained on ammonium sulfate treatment was not changed by repeated isoelectric separations.

The spleen pI 7.8-isozyme fraction obtained by isoelectric separation after ammonium sulfate treatment was mixed with an equal volume of 0.2 mM fructose 1,6-diphosphate, and the resulting mixture (10 ml) was again subjected to isoelectric separation (Fig. 13).

Fig. 13

It was found that the pI 7.8-isozyme was converted into pI 6.2- and pI 6.6-isozymes. In some experiments, only pI 6.6-isozyme was formed. The extent of the conversion increased with increasing concentrations of fructose 1,6-diphosphate. If the isoelectric separation was prolonged, the pI 6.2-isozyme initially formed was gradually

converted into pI 6.6-isozyme, but yielded little pI 7.8-isozyme. In addition, it was found that pI 7.8-isozyme was mostly converted into pI 6.2-isozyme when 1 mM fructose 1,6-diphosphate was added to the whole of the solution placed in the electrofocusing column.

These results indicate that the three pI-isozymes present in spleen extracts are identical in protein species (spleen-type isoenzyme), and that this isoenzyme, like the liver-type isoenzyme, probably possesses two kinds of sites capable of binding with fructose 1,6-diphosphate; pI 6.6- and pI 6.2-isozymes are types bound with fructose 1,6-diphosphate at one and two kinds of sites, respectively, and pI 7.8-isoenzyme is a type free of the sugar phosphate.

1-c) Muscle: When extracts from skeletal muscles of normal rats were subjected to isoelectric separation, only pI 7.4-isozyme was detectable (muscle-type isoenzyme). The muscle pI 7.4-isozyme was not influenced by repeated isoelectric separations before and after ammonium sulfate treatment or with and without addition of fructose 1,6-diphosphate. Therefore, it is conceivable that the pI 7.4-isozyme possesses no kind of site capable of binding with fructose 1,6-diphosphate.

1-d) Other Normal Tissues: It was previously reported that extracts from kidney, lung and erythrocytes of normal rats also contain pI 7.8-isozyme with pI 6.2- and/or pI 6.6-isozymes.[14] When these extracts were subjected to ammonium sulfate treatment and then to isoelectric separation, most of the pI 6.2-isozyme in the kidney

extracts and all of the pI 6.6-isozyme in the lung and erythrocyte extracts were converted into pI 7.8-isozyme.

1-e) Rhodamine sarcoma: When extracts from Rhodamine sarcoma were subjected to isoelectric separation, the pyruvate kinase was divided into pI 6.2-, pI 7.4- and pI 7.8-isozymes; the percentage activities were 15 ± 5%, 7 ± 5% and 78 ± 5%, respectively. With respect to the behavior in isoelectric separation, the pI 7.4-isozyme was identical to muscle pI 7.4-isozyme, and the pI 6.2- and pI 7.8-isozymes were identical to spleen pI 6.2-, pI 6.6- and pI 7.8-isozymes.

The amounts of pI-isozymes present in the extracts from various tissues, previously subjected to ammonium sulfate treatment, are summarized in Table IV. The yields of the total activities amounted

Table IV

to 85 ± 10%.

IV-2. Some Properties of Various pI-Isozymes of Pyruvate Kinase

The results described above conclude that pyruvate kinase in various tissues of rats, if they are free of fructose 1,6-diphosphate, are classified into the three types of isoenzymes, liver-type (pI 6.2), muscle-type (pI 7.4) and spleen-type (pI 7.8). This conclusion agrees with that by Tanaka *et al.*[32,33,36,39] who called spleen-type as type M_2 on the basis of the cross reaction of the antibody for the muscle-type isoenzyme to the spleen-type isoenzyme, but not on the

basis of the organ which contains the isoenzyme most abundantly.

Furthermore, these pI-isozymes obtained from extracts of livers, spleens, skeletal muscles and Rhodamine sarcoma by isoelectric separation before and after ammonium sulfate treatment were examined and compared on various properties, such as molecular weight, kinetics and binding property with fructose 1,6-diphosphate.[42] The molecular weight of spleen pI 7.8-isozyme was estimated to be approximately 110,000 by molecular-sieve fractionation. However, this value is half of that estimated by imamura et al.[39] (M.W. 216,000). Recently, Ibsen and Trippet[43] reported that pI-isozymes of pyruvate kinase from rat kidney are ultracentrifugally separable into two species different in molecular weight; their molecular weight are 178,000 and 116,000.

Nagao et al. have recently found, using spleen pI 7.8-isozyme purified completely from Rhodamine sarcoma, that this isozyme is a dimer, which is composed of the same subunit having a molecular weight of 60,000 (to be published). In addition, they have directly demonstrated, using ^{14}C-fructose 1,6-diphosphate, that the spleen-type isoenzyme of pyruvate kinase has two kinds of sites capable of binding with fructose 1,6-diphosphate, and that these two kinds of sites are considerably different in an ability to bind with the sugar phosphate.

IV-3. Effect of Growth of Rhodamine sarcoma on Pyruvate Kinase pI-Isozymes in Liver Nakamura et al.[14] found, using the isoelectric separation method, that when Rhodamine sarcoma is transplanted in

rats, the total activity of pyruvate kinase in the livers increases to a significant extent; pI 6.2-isozyme content increases, whereas other pI-isozymes are hardly affected. Their results were reproduced in the present study (Fig. 14). When extracts from livers of

Fig. 14

Rhodamine sarcoma-bearing rats were subjected to ammonium sulfate treatment and then to isoelectric separation, the pI 5.4-isozyme disappeared, while the pI 7.8-isozyme content increased to a remarkable extent. The results are summarized in Table IV. These data indicate that the increase of total activity of pyruvate kinase in the livers of rats on tumor transplantation was mostly due to the increase of the content of spleen pI 6.2-isozyme but not liver pI 6.2-isozyme.

IV-4. Effect of Injection into Rats of Nuclei from Rhodamine sarcoma or Chromatins from the Tumor, Spleen and Liver on Pyruvate Kinase pI-Isozymes in Livers It was previously reported that when chromatin prepared from Rhodamine sarcoma is injected into rats, the total activity of pyruvate kinase in the livers increases in the same way as when the tumor grows in rats.[14] In the case of injection of sarcoma chromatin, the pI 7.8-isozyme content increases to a significant extent, unlike the case with tumor-bearing rats.

In the present study, nuclei and chromatin were prepared from Rhodamine sarcoma, and from spleens and livers of normal rats as

well as the tumor tissue, respecively, and the effect of their injections into rats on pI-isozymes in liver pyruvate kinase was examined according to the method of Nakamura et al.[14)]

When the sarcoma nucleus preparation was injected into rats weighing 120-140 g at three different doses, 14, 28 and 56 mg in protein, and the extracts from the livers were subjected to isoelectric separation after ammonium sulfate treatment, the relative content of spleen pI 7.8-isozyme in the extracts increased remarkably with increasing doses, whereas that of liver pI 6.2-isozyme decreased slightly (Table V). Since the total activity of pyruvate

Table V

kinase in the extracts also increased to a significant extent with increasing doses, the actual content of the liver pI 6.2-isozyme in the extracts hardly changed, while that of the spleen pI 7.8-isozyme in the extract from rats injected at the maximum dose was more than two times the level in the extract from control rats. In addition, it was found that when the sarcoma nucleus preparation was repeatedly injected into rats every 24 hr at a dose of 20 mg in protein, the total activity of pyruvate kinase and the relative activity of spleen pI 7.8-isozyme in the liver extracts increased continuously to remarkable extents, respectively. The actual content of the spleen pI 7.8-isozyme in the extract from rats injected three times with the nucleus preparation was more than three times the

level in the extract from control rats, whereas that of liver pI 6.2-isozyme did not change significantly. Thus increase of the spleen-type isoenzyme content in the liver extracts from rats injected with the sarcoma nucleus preparation was nicely in contrast to the decrease of catalase activity level in the extracts as shown in Table V.

In order to confirm the fact obtained by Nakamura et al.,[14] the chromatin preparations from Rhodamine sarcoma, spleen and liver were injected once into rats at a dose of 20 mg in protein, and the liver extracts were subjected to ammonium sulfate treatment and then to isoelectric separation. The results are summarized in Table IV. In the case of tumor-bearing rats and rats injected with the sarcoma chromatin preparation, the spleen pI 7.8-isozyme content increased to four to six times the levels in extracts from livers of normal rats, whereas the liver pI 6.2-isozyme content decreased slightly. The same kind of experiment was carried out with the chromatin preparations from spleens and livers of normal rats. It was found that when the spleen chromatin preparation was injected into rats, and the liver extract was subjected to ammonium sulfate treatment and then to isoelectric separation, the spleen pI 7.8-isozyme content in the livers increased appreciably. The injection of the liver chromatin preparation did not cause any change in the pI-isozyme pattern.

These results, in good accord with the previous findings, indicate that chromatin from Rhodamine sarcoma contains a substance

capable of increasing the spleen-type isoenzyme of pyruvate kinase in liver besides the catalase-depressing substance, and that the effective substance is present also in spleen chromatin in somewhat lower content than in sarcoma chromatin, but barely or not at all in liver chromatin.

V. Localization of *in vivo* Liver Catalase-depressing Substance in Chromatin Prepared from Rhodamine sarcoma

Chromatin was prepared from Rhodamine sarcoma of rats by the method described above. In most of the chromatin preparations thus obtained, the ratio in amount of the protein to the DNA was 3 to 4 (Table VI). This value is somewhat higher than the value

Table VI

reported by other workers,[44] who analyzed chromatin preparations from other tissues of rats. The chromatin preparation, if injected into mice, decreased the catalase activities of the livers to a significant extent (Fig. 15).

Fig. 15

The chromatin prepartion from Rhodamine sarcoma was fractionated into three major components; histone, non-histone protein, and DNA. The resulting fractions were assayed for the *in vivo* liver catalase-depressing activity.

V-1. Histone Histone was extracted from chromatin with 0.1 M H_2SO_4 as described above, and the acid-insoluble substance was saved as the histone-free chromatin (Table VI). The resulting extract (histone in Table VI) barely contained DNA or RNA. When the extract was subjected to isoelectric separation, the substance showing absorbance at 280 nm formed a single, sharp peak of pI = 9.5 (Fig. 16). This indicated that the extract contained only

Fig. 16

alkaline proteins (histone). The fractions with pI values centered at 9.5 were collected and mixed. The resulting mixture was used as the histone preparation. The histone preparation showed *in vivo* liver catalase-depressing activity, the extent of which increased with increasing doses (Fig. 17). It is known that histone is composed

Fig. 17

of some basic proteins rich in L-lysine as well as L-arginine. Therefore, the depressing activity was measured with poly-L-lysine and with L-lysine. Poly-L-lysine showed the depressing activity to practically the same extent as the histone preparation, but not L-lysine.

The histone-free chromatin was also subjected to assay of *in vivo* liver catalase-depressing activity (Fig. 15). Despite the

lack of histone, it showed the depressing activity to almost the same extent as the whole chromatin preparation. This suggests that a depressing substance other than histone was present in Rhodamine sarcoma chromatin, and that the *in vivo* liver catalase-depressing activity of the former was significantly higher than that of the latter.

V-2. Non-histone Protein and DNA Non-histone protein and DNA were prepared from the chromatin of Rhodamine sarcoma by the procedure schematically shown in Fig. 18. A chromatin preparation was

Fig. 18

dissolved in 2 M NaCl and the resulting solution was subjected to molecular-sieve fractionation on a Sephadex G-200 column previously equilibrated with 2 M NaCl. In the presence of such a high concentration of the salt, the proteins were almost completely dissociated from DNA; thus, DNA having higher molecular weights than the exclusion limit of the Sephadex (M.W. approximately 200,000) was eluted at a void volume of the column and proteins were mostly eluted later (Fig. 19). However, a small amount of protein was still bound

Fig. 19

with DNA, and the complex of DNA and protein was eluted in DNA fractions. The DNA fractions were mixed and the mixture was supple-

mented with such an amount of CsCl that the salt concentration would be 4 M, followed by centrifugation at 105,000 g for 16 hr; DNA was precipitated forming a pellet on the bottom of the centrifuge tubes, whereas the protein formed a thin white film floating on the surface of the CsCl solution. The precipitated DNA was collected and desalted (protein-free DNA preparation). On the other hand, the protein fractions collected by the molecular-sieve fractionation contained non-histone protein and histone, being practically free of DNA. On the basis of their molecular weights, it seems likely that non-histone protein is mostly eluted in the fraction numbers from 45 to 80 (peak at No. 70), and histone in the fraction numbers from 85 to 110 (shoulder). Fractions for both non-histone protein and histone (fraction numbers from 55 to 105) were mixed. The mixture was concentrated-desalted by ultrafiltration through a PM-10 membrane filter (exclusion limit in molecular weight, approximately 10,000) with the use of an Amicon Diaflo apparatus. The concentrated-desalted sample was subjected to isoelectric separation (Fig. 20). Contrary to expectation, it was found that the

Fig. 20

content of histone was significantly lowered by the filtration. Doubtless, histone passed through the membrane filter because of its lower molecular weight and fibrous molecular form. As expected, the pI-$A_{280\ nm}$ curve had several peaks in a weakly acidic region.

The acidic protein was divided roughly into two groups; one with lower pI than 4 and the other from pI 4 to 7. These two groups of non-histone protein were separately dialyzed against water. The dialyzed samples are called F-1 (pI<4) and F-2 (pI 4-7). These two non-histone protein samples and the protein-free DNA preparation were subjected to assay of in vivo liver catalase-depressing activity (Fig. 21). Only F-2 showed the depressing activity to a

Fig. 21

significant extent at a dose of 0.3 mg, and neither F-1 nor DNA even at higher doses.

Absorbance spectra were measured with the whole chromatin, the protein-free DNA preparation, and the non-histone protein preparation (Fig. 22). The protein-free DNA preparation showed a peak

Fig. 22

at 260 nm and a trough at 233 nm with a ratio of the $A_{260\ nm}$ to the $A_{230\ nm}$ of 2.6 (Fig. 22-b). The whole chromatin preparation showed a peak at 258 nm and a trough at 239 nm, with the ratio = 0.92 (Fig. 22-a); this value is somewhat lower than that with calf thymus chromatin,[45] but it increased to 1.3-1.4 when whole chromatins were extracted with 2 M NaCl. The absorbance spectrum of the whole chromatin preparation resembled that of the effective substance

isolated by Matuo.[10] The non-histone protein preparation showed a peak at 280 nm without a hint of a peak at 260 nm (Fig. 22-c). This spectrum resembles that of non-histone protein prepared from rat liver.[46]

VI. Isolation of *in vivo* Liver Catalase-depressing Substance from Nucleus Preparation from Rhodamine sarcoma Free of Endogeneous Proteolytic Activity

VI-1. Heat Treatment of Nucleus Preparation from Rhodamine sarcoma It was reported by some workers[47-49] that some chromatin preparations contained a trypsin-like protease which digested histone and non-histone protein to a remarkably extent. In addition, Matuo *et al*.[11] have urged the necessity of caution against the proteolytic enzyme present in nuclear fraction of Rhodamine sarcoma, which hydrolyzes *in vivo* liver catalase-depressing substance into inactive peptides. In the present study, when the chromatin preparation from Rhodamine sarcoma was incubated at 37°, the chromosomal proteins and DNA were significantly hydrolyzed to small fragments soluble in 5% trichloric acid, indicating that the chromatin preparation contained significant amounts of endogeneous protease and deoxyribonuclease (Fig. 23). As expected, sodium citrate inhibited

Fig. 23

considerably the deoxyribonuclease activity, while it activated the proteolytic activity to a significant extent. The protease bound

with chromatin was completely inactivated by heating at 90° for 3 min, but hardly or not at all by adding either soybean trypsin inhibitor or sodium bisulfite, differing from the finding by Panyim *et al.*[47] that 5 m*M* sodium bisulfite inhibited the activity of protease bound with calf thymus chromatin (Fig. 24). The storage

Fig. 24

of chromatin preparation at 0° seems not sufficient to prevent it from the proteolysis. On the other hand, Matuo *et al.*[11] showed that *in vivo* liver catalase-depressing substance present in nuclear fraction of Rhodamine sarcoma is stable against heating at 100° for 1 hr. On the basis of these facts, it was tried in the present study to isolate original form of *in vivo* liver catalase-depressing substance from nuclei of Rhodamine sarcoma after heat treatment to inactivate the endogeneous protease. The procedure for the isolation of *in vivo* liver catalase-depressing substance, which somewhat differs from that shown in Fig. 18, was schematically shown in Fig. 25.

Fig. 25

The nucleus preparation from Rhodamine sarcoma was heated in a boiling water bath for 10 min in order to inactivate completely the protease, and then washed with a mixture of chloroform and

methanol (2:1) according to the method developed by Matuo,[10] who demonstrated that the washing of crude nuclear fraction with the mixture facilitated the solubilization of *in vivo* liver catalase-depressing substance. The washed nucleus preparation was dried in vacuum. The resulting dried powder was suspended in 100 volumes (v/w) of water with the aid of a Waring blendor and extracted for 24 hr at 4° under continuous stirring, followed by centrifugation at 105,000 *g* for 1 hr. The extraction was repeated once more. The combined extracts containing chromatin were dialyzed against water and then lyophilized, followed by dissolving in 0.01 *M* sodium phosphate buffer (pH 7.0) to make the protein concentration to approximately 1 mg/ml. Insoluble material which occured in trace was removed by centrifugation at 105,000 *g* for 1 hr.

Matuo *et al.*[11] found that when water-soluble extract from particular fraction of Rhodamine sarcoma was incubated at pH 7.0 and 30° for longer than 19 hr, the amount of precipitate (pH 4-ppt) which was obtained by adjusting pH of the solution to 4.0 decreased significantly. They concluded that the decrease of pH 4-ppt was due to the extent of segmentation of the substances present in the extract by proteolytic activity as contaminant. When a chromatin solution from the heated nucleus preparation of Rhodamine sarcoma was incubated at pH 7.0 and 30° for various lengths of time, and then subjected to pH 4 treatment, the chromatin content in the supernatant (pH 4-supe) hardly changed even after the incubation for 40 hr (Table VII). This result suggests

Table VII

that the proteolytic activity was almost completely inactivated during the heat treatment of the nucleus preparation.

VI-2. Separation of Nucleic Acids and Protein in pH 4-Ppt For further purification of *in vivo* liver catalase-depressing substance, pH 4-ppt was used as a starting material. Although pH 4-supe also has the activity, this fraction seems to contain mainly active fragments which were formed by the protease.[11] The pH 4-ppt from heated nucleus preparation was analyzed to contain 74% protein, 17% DNA and 8% RNA. The pH 4-ppt was dissolved in 0.01 *M* sodium phosphate buffer (pH 7.0), followed by centrifugation at 105,000 *g* for 1 hr. The resulting supernatant was supplemented with such an amount of CsCl that the salt concentration would be 4 *M*, followed by centrifugation at 105,000 *g* for 16 hr. The tube content was divided into 5 fractions and the amounts of protein, DNA and RNA were measured with these fractions (Fig. 26). DNA and RNA were

Fig. 26

precipitated on the bottom of the tube, whereas most of protein formed floating layer on the surface. The upper one-third of the tube content was collected and used as the protein fraction. Both DNA and protein fractions were dialyzed against 0.01 *M* sodium phosphate buffer (pH 7.0) and then subjected to the assay of *in vivo* liver catalase-depressing activity (Fig. 27) The protein

Fig. 27

fraction showed the depressing activity as pH 4-ppt did, but not DNA fraction. Consequently, the protein fraction was subjected to further fractionations after removing insoluble material by centrifugation at 105,000 g for 1 hr. This supernatant contained only negligible amounts of DNA and RNA, and its absorption spectrum had a maximum at 277 nm.

VI-3. Molecular-sieve Fractionation The solution of nucleic acids-free protein was fractionated by molecular-sieving on a Sephadex G-200 column (exclusion limit in molecular weight of dextran, approximately 200,000)(Fig. 28). Fractions around main peak (No.53 to No.63)

Fig. 28

were collected and concentrated by means of lyophilization. The concentrated protein fraction, which showed *in vivo* liver catalase-depressing activity (Fig. 29), was subjected to 2nd molecular-sieve

Fig. 29

fractionation on a Sephadex G-200 column (Fig. 28). The molecular weight of this protein fraction was estimated to be approximately 60,000 (P-60,000), according to the method of Andrews[50] using the same molecular weight marker proteins as those described by Matuo *et al.*[11] Contrary to the result with the use of samples without heat-treatment,[11] further segmentation hardly occured. The fractions around the peak were

again collected and subjected to 3rd molecular-sieve fractionation on a column of Sephadex G-100 (exclusion limit in molecular weight of dextran, approximately 100,000) (Fig. 30). Single peak was

Fig. 30

again obtained. The molecular weight of approximately 60,000 was again calculated from the result with Sephadex G-100. It was confirmed by these results that the proteolytic activity in the sample was almost completely inactivated by the prior heat treatment.

VI-4. Isoelectric Separation The main fractions from 2nd molecular-sieve fractionation on Sephadex G-200 were collected, concentrated by lyophilization and desalted by dialysis. The resulting sample was subjected to isoelectric separation with Ampholine carrier ampholytes capable of producing a gradient of pH from 3 to 10 (1st isoelectric separation)(Fig. 31). Main peak of

Fig. 31

protein was obtained at around pH 5.0, which showed *in vivo* liver catalase depressing activity (P-pI 5.0)(Fig. 29). Fractions around main peak (No.10 to No.13) were collected, desalted by means of dialysis and subjected to 2nd isoelectric separation with Ampholine carrier ampholytes capable of producing a gradient of pH from 4 to 6 (Fig. 32). Single peak at pH 5.0 with a slight shoulder at

Fig. 32

higher pH range was obtained. When the peak fraction was subjected to molecular-sieve fractionation on a Sephadex G-100 column, the same single peak was obtained as in Fig. 30. Thus purified protein migrated in SDS-polyacrylamide gel electrophoresis, forming a single band of protein. The molecular weight of the band was estimated to be approximately 60,000, indicating that this is a primer protein. The purified protein had absorption peak at 277 nm, excluding the contamination with nucleic acids. Approximately 500 g in wet weight of Rhodamine sarcoma tissue yielded approximately 60 mg of purified protein.

DISCUSSION

It was previously reported by Kannan *et al.*[13] that the nuclear fractions from Rhodamine sarcoma and from dorsal muscles of normal rats, but hardly the nuclear fraction from livers of normal rats, depress the activity of liver catalase to a significant extent when the fractions have been injected into mice. In the present study, nuclei were isolated from brains, kidneys, and spleens of normal rats, as well as livers of normal rats and Rhodamine sarcoma, and the *in vivo* liver catalase-depressing activities of these nuclei were compared to one another. In a good accordance with the report of Kannan *et al.*,[13] it was found that nuclei from various tissues of normal rats besides those from Rhodamine sarcoma, showed more or less the depressing activity, indicating that an *in vivo* liver catalase-depressing substance was present also in normal tissues (Table II). However, it is conceivable that the contents of the depressing substance were different for the kinds of tissues from which nuclei were isolated; the depressing activities were high in nuclei from Rhodamine sarcoma and brain, moderate in those from kidney and spleen, and low in those from liver. On the other hand, when the catalase activities of various kinds of tissues of rats were measured without injection of nuclei, the activities were low in Rhodamine sarcoma and brain, moderate in kidney and spleen, and high in liver (Table III). Together with the findings by Kannan *et al.*[13] with muscle, which is low in catalase activity, the present findings indicate that the catalase

activities of various tissues, whether normal or malignant, were in contrast to the extents of *in vivo* liver catalase-depressing activities in the nuclei prepared therefrom. Earlier, Riley[51] demonstrated that homogenates of livers of normal mice, when injected into mice, decreased the catalase activities of the livers. Later, Ohashi[24] reported that spleens and livers of normal mice contained the depressing substance, but in a small amount. Their findings were in accordance with the present findings.

It was found that when sarcoma nuclei were injected into mice, the activity and the amount of catalase in the liver were decreased in parallel (Fig. 5), and that this decrease became markedly greater when the injection was repeated daily (Fig. 1). In addition, the previous finding that sarcoma nuclei, when injected into mice, decrease the liver catalase activity to much higher extent than liver nuclei was reproduced in the daily repeated injections. These results indicate that the effective substance present in sarcoma nuclei, if injected into mice, decreases catalase activity level in the livers by depressing amount of catalase protein, but not by inactivating the enzyme activity.

Doubtless, the catalase activity level in the liver was maintained nearly constant by the balance of biosynthesis and degradation of catalase. It is conceivable that the biosynthesis of catalase was inhibited by the injection of sarcoma nuclei, on the basis of the results obtained by injection of sarcoma nuclei or allyisopropylacetamide, or both (Fig. 8). This is in accordance with Hozumi and

Sugimura.[30] However, it is still uncertain whether the injection of sarcoma nuclei stimulated the degradation of catalase to a slight extent or not.

Higashi and Shibata,[52] and Holmes and Masters[53] reported that catalase in the soluble fraction and catalase in the particulate fraction are different in nature. Nakamura et al.[54] found that free catalase and particle-bound catalase are different in their life time. It was found in the present study with mice that when liver catalase was irreversibly inhibited by injection of amino-triazole, the restoration of the particle-bound catalase in the liver was significantly faster than that of the free catalase (Fig. 7), and that when sarcoma nuclei were injected, the free catalase in the liver decreased to a significantly higher extent than the particle-bound catalase (Fig. 3). It is conceivable that the particle-bound catalase is synthesized at a faster rate than the free catalase, and/or the former is degraded at a slower rate than the latter. The difference in the sensitivity of both catalase to the injected substance may be due to the difference in the turnover rate, or in the mechanisms of biosynthesis and in vivo degradation. It is difficult now to explain more sufficiently this result.

Multiple forms of pyruvate kinase have been observed in various tissues of animals such as rat liver,[14,32,33] mouse liver,[55] rat kidney[56] and rabbit muscle.[57] Hess et al.[41] observed, using the same isoelectric separation method as that in the present study, that the pyruvate kinase in pig liver is separable into pI 5.3-

isozyme and pI 6.1-isozyme, and found that pI 6.1-isozyme, when bound with fructose 1,6-diphosphate, is converted into pI 5.3-isozyme.

In the present study, it was concluded that pyruvate kinase in various tissues of rats, if it was free of fructose 1,6-diphosphate, could be classified into the three types of isoenzymes, liver-type (pI 6.2), muscle-type (pI 7.4) and spleen-type (pI 7.8). This conclusion agrees with that by Tanaka et al., [32,33,36,39] who called spleen-type as type M_2. Since liver-type and spleen-type isoenzymes,but not muscle-type isoenzyme, were separable into the three pI-isozymes by isoelectric separation (liver pI 5.4-, pI 5.6- and pI 6.2-isozymes and spleen pI 6.2-, pI 6.6- and pI 7.8-isozymes), respectively, both isoenzymes appeared to possess two kinds of sites capable of binding with fructose 1,6-diphosphate. From the behaviour of these pI-isozymes in repeated isoelectric separation with and without previous ammonium sulfate treatment or previous incubation with fructose 1,6-diphosphate (Fig. 9-13), it seems likely that the two kinds of the sites are not equivalent to each other with respect to the ability to bind with fructose 1,6-diphosphate. Recently, Muroya et al.[42] and Nagao et al. (to be published) confirmed this result, using spleen-type isoenzyme purified from Rhodamine sarcoma, and found that the binding of fructose 1,6-diphosphate at both kinds of the sites was regulated by a balance between the concentration of the sugar phosphate and the salt concentration (ionic strength) in the solution containing the isoenzymes. Studies on the quantitative

analysis of fructose 1,6-diphosphate bound with the spleen-type pI 6.2- and pI 6.6-isozymes, and the protein chemistry of the three isoenzymes are now under investigation.

In Rhodamine sarcoma-bearing rats, it was found that in their livers, spleen-type isoenzyme of pyruvate kinase increased to a remarkable extent (Fig. 14 and Table IV). In addition, when either nucleus or chromatin preparation from Rhodamine sarcoma was injected into rats, the isoenzyme increased in their livers to an extent similar to that in Rhodamine sarcoma-bearing rats, in accordance with the previous finding.[14)] When chromatin prepared from spleens of rats was injected into rats, the isoenzyme increased in their livers to an appreciable extent, although the extent was much lower than in the case with sarcoma chromatin. These results suggest that chromatins of Rhodamine sarcoma and spleen, but barely or not at all spleen chromatin, contain the substance capable of increasing the spleen-type isoenzyme in liver as well as the *in vivo* liver catalase-depressing substance. It is still uncertain whether both effective substances in the chromatins are identical to each other or not.

The localization of *in vivo* liver catalase-depressing substance was studied in chromatin prepared from Rhodamine sarcoma. The chromatin was fractionated into DNA, histone, and non-histone protein. Of these major components of chromatin, the histone fraction and the non-histone protein fraction showed *in vivo* liver catalase-depressing activity, but not the DNA fraction (Fig. 15,17,21). However, it was

found that the depressing activity was significantly higher in the non-histone protein fraction than in the histone fraction. Since it is known that histone is basic proteins rich in L-lysine residue, poly-L-lysine and L-lysine were examined for *in vivo* liver catalase-depressing activity. Poly-L-lysine, but not L-lysine, showed the depressing activity to an extent similar to that of the histone fraction (Fig. 17). Aoki *et al.*[58] described that histone preparations from Walker carcinosarcoma and calf thymus, when injected into mice, decreased the catalase and tryptophan pyrrolase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12) activities of the livers, but not non-histone protein preparations from the tumor nuclei. In addition, they fractionated the histone preparations and found that the histone F-1 richest in the content of L-lysine residue among the fractions was most effective in depressing the activities of both enzymes. Their findings are in accordance with the present findings with respect to histone, whereas there is a discrepancy between their and our findings in respect to non-histone protein. It has been currently accepted, however, that histone is widely distributed in animals and higher plants in the state bound to chromatin and that there is no significant difference among various tissues in animals.[59] The similarity in extent of *in vivo* liver catalase-depressing activity between the histone fraction and poly-L-lysine suggests that such a low *in vivo* liver catalase-depressing activity of the nuclei isolated from livers of normal animals was at least in most part brought about by the histone. When the non-

histone protein prepared from chromatin of Rhodamine sarcoma was further fractionated by isoelectric fractionation, the fraction of pI 4-7 showed a high *in vivo* liver catalase-depressing activity, but not the fraction of pI<4 (Fig. 21).

On the other hand, it was previously reported by Matuo *et al.*[11] that extract from crude nuclear fraction of Rhodamine sarcoma contained protease which hydrolyzed *in vivo* liver catalase-depressing substance to inactive peptides. According to their finding, it was tried in the present study to isolate original form of *in vivo* liver catalase-depressing substance from Rhodamine sarcoma nuclei. In this attempt, a single protein was isolated from Rhodamine sarcoma nuclei free of proteolytic activity. The pure protein, which had a molecular weight of 60,000 and a pI value of 5.0, showed *in vivo* liver catalase-depressing activity when it was injected into mice. It seems rational to conclude, therefore, that *in vivo* liver catalase-depressing activities of nuclei isolated from Rhodamine sarcoma, muscle,brain, kidney, and spleen, which exceed the depressing activity of nuclei isolated from liver, are brought about by the acidic protein; higher the content of the acidic protein, higher the *in vivo* liver catalase-depressing activity of the nuclei. In recent years, great attentions have been focused on functions of histone and non-histone protein present in the chromatins as regulators of the gene expression in animal cells. The facts described above, together with the present findings, suggest a possibility that alterations of liver enzymes invoked by tumor bearing may be brought about by the

corresponding kinds of non-histone protein present in the chromatin of the tumor cells, which are liberated from intact or broken cells of the tumor, in the same manner as the gene expression is differently regulated in various tissues of normal animals. Perhaps, the manner for the regulation is not so many in number as the variety of the enzymes present in cells because of the limitation by the kinds of non-histone protein.

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Table I. Chemical Compositions of Nuclei from Various Tissues of Rats

Source[a]	Relative weight in nucleus preparation		
	DNA	RNA[b]	Protein
Liver	(1.0)	0.21	2.7
Kidney	(1.0)	0.10	2.4
Spleen	(1.0)	0.04	1.9
Brain	(1.0)	0.14	3.4
Rhodamine sarcoma	(1.0)	0.29	3.9

a) Tissues other than Rhodamine sarcoma were obtained from normal rats.

b) Values normalized for the DNA content.

Table II. *In vivo* Liver Catalase-depressing Activities of Nuclei Isolated from Various Tissues of Rats

Injected nuclei[a]															
Rhodamine sarcoma			Brain			Kidney			Spleen			Liver			
Dose (mg protein/mg DNA)	Mouse No.	Catalase activity K_{obs} x 100 (%)[b]	Dose (mg protein/mg DNA)	Mouse No.	Catalase activity K_{obs} x 100 (%)[b]	Dose (mg protein/mg DNA)	Mouse No.	Catalase activity K_{obs} x 100 (%)[b]	Dose (mg protein/mg DNA)	Mouse No.	Catalase activity K_{obs} x 100 (%)[b]	Dose (mg protein/mg DNA)	Mouse No.	Catalase activity K_{obs} x 100 (%)[b]	
(0)	1	3.22	(0)	1	3.87	(0)	1	3.70	(0)	1	3.87	(0)	1	2.67	
	2	3.00		2	3.94		2	3.81		2	3.94		2	2.68	
	3	3.09		3	3.98		3	4.17		3	3.98		3	3.17	
	4	3.19		4	3.37		4	3.11		4	3.37		4	3.11	
				5	3.41					5	3.41		5	3.52	
	Av.	3.13	0	Av.	3.71	0	Av.	3.70	0	Av.	3.71	0	Av.	3.03	0
(1.8 / 0.45)	1	2.73	(1.5 / 0.45)	1	3.01	(1.1 / 0.45)	1	3.42	(0.9 / 0.45)	1	3.55	(1.2 / 0.45)	1	2.90	
	2	2.45		2	3.10		2	3.75		2	3.73		2	3.37	
	3	2.62		3	3.47		3	3.57		3	3.93		3	3.06	
	Av.	2.60	17	Av.	3.19	14	Av.	3.58	3	Av.	3.74	-1	Av.	3.11	-3
(7.0 / 1.8)	1	2.55	(6.1 / 1.8)	1	3.37	(4.3 / 1.8)	1	2.99	(3.4 / 1.8)	1	3.68	(4.9 / 1.8)	1	2.93	
	2	2.36		2	2.98		2	3.44		2	2.98		2	2.94	
	3	2.44		3	2.94		3	2.96		3	3.38		3	2.85	
	Av.	2.45	22	Av.	3.10	16	Av.	3.13	15	Av.	3.35	10	Av.	2.91	4
(18.0 / 4.5)	1	2.18	(15.3 / 4.5)	1	2.68	(10.8 / 4.5)	1	3.03	(8.6 / 4.5)	1	3.41	(12.3 / 4.5)	1	2.72	
	2	1.93		2	2.89		2	3.57		2	2.98		2	2.78	
	3	1.97		3	2.69		3	2.59		3	3.01		3	2.86	
	Av.	2.02	35	Av.	2.75	26	Av.	3.06	17	Av.	3.13	16	Av.	2.79	8

a) Tissues other than Rhodamine sarcoma were obtained from normal rats.

b) Percentage depression of catalase activity.

Table III. Catalase Activities of Various Tissues of Rats

Tissue[a)]	Rat No.	Catalase activity of tissue K_{obs} x 100	%
Liver	1	4.1	
	2	3.7	
	3	3.5	
	Average	3.7	(100)
Kidney	1	1.5	
	2	1.5	
	3	1.3	
	Average	1.4	39
Spleen	1	0.28	
	2	0.26	
	3	0.33	
	Average	0.29	8
Brain	1	0.01	
	2	0.02	
	3	0.02	
	Average	0.02	0.5
Rhodamine sarcoma	1	0.03	
	2	0.03	
	3	0.03	
	Average	0.03	0.8

a) Tissues other than Rhodamine sarcoma were obtained from normal rats.

Table IV. Amounts of pI-Isozymes in Extracts of Various Tissues After Conversion into Their Fructose 1,6-diphosphate-free Forms by Ammonium Sulfate Treatment

Rats	Diet	Tissues	Specific activities (units/g protein[a])[b]		
			pI 6.2-isozyme (Liver-type)	pI 7.4-isozyme (Muscle-type)	pI 7.8-isozyme (Spleen type) (M_2)
Normal	Ordinary	Liver	190	14	36
"	High carbohydrate	"	657	14	42
Rh.sarcoma-bearing	Ordinary	"	164	20	196
Injected with Rh.sarcoma chromatin	"	"	155	30	145
Injected with spleen chromatin	"	"	173	24	87
Rh.sarcoma-bearing	"	Rh.sarcoma	0	140	1,260
Normal	"	Spleen	0	73	537
"	"	Lung	0	86	524
"	"	Kidney	50	67	443
"	"	Erythrocyte	3	1	17
"	"	Skeletal muscle	0	8,900	0
"	"	Heart muscle	0	950	0
"	"	Brain	0	1,900	0

a) The amount of protein present in extracts before ammonium sulfate treatment.

b) The total units present in extracts from the tissues was proportionally allotted on the basis of the relative activities of the fractions for pI 6.2-, pI 7.4- and pI 7.8-isozymes obtained by isoelectric separation of the extracts.

Table V. Effect of Injection into Rats of Rhodamine sarcoma Nuclei on Relative Activities of Pyruvate Kinase Isoenzymes and Catalase Activity in Livers

Rats[a)] (130±10g) injected with	Dose (mg protein)	Injection times	Pyruvate kinase activity in liver extract U/ml	Recovery in isoelectric separation (%)	Relative activities of Pyruvate Kinase isoenzymes (%)			Catalase activity	
					Liver-type (L)	Muscle-type (M)	Spleen-type (M_2)	$K_{obs} \times 10^2$	%
Saline	0	1	9.2	77	67	8	25	3.19	(100)
Rh.sarcoma nuclei	14	1	10.5	67	65	8	27	2.54	80
"	28	1	10.5	67	62	6	32	2.28	71
"	56	1	12.3	88	49	8	43	1.78	56
Saline	0	1	8.4	62	70	5	25	3.98	(100)
Rh.sarcoma nuclei	20	1	10.9	65	65	6	29	3.37	85
"	20	2	11.6	68	59	6	35	2.53	63
"	20	3	12.5	61	40	8	52	2.00	50

a) Three rats were used in each experiment, and the extracts from their three livers were mixed and subjected to isoelectric separation after ammonium sulfate treatment. For the assay of catalase activity, a small portion was removed from each of the livers and used as described above. The average value of catalase activity in the three homogenates was shown.

Table VI. Components of Chromatin Preparations from Rhodamine Sarcoma

Component	Chromatin (%)	Histone (%)	Histone-free chromatin (%)
DNA	(100)	0	82
RNA	7	0.3	6
Protein	310	164	146

Table VII. Effect of Incubation at 30° of Chromatin Extracted from Heated Nucleus Preparation of Rhodamine sarcoma on Its Degradation

Incubation time[a)] (hr)	$A_{258\ nm}$ of pH 4-supe[b)] (%)
0	51
15	50
40	54

a) Aliquots of crude chromatin extract were incubated at pH 7.0 and 30° for various lengths of time as indicated.

b) After the incubation, pH was adjusted to 4.0 by adding 2 N acetic acid, followed by centrifugation. Absorbance at 258 nm of supernatant was measured.

Fig. 1. Effect of injection of Rhodamine sarcoma nuclei into mice on liver catalase activity

Rhodamine sarcoma nuclei (4.8 mg protein/30 g body weight) were injected into mice weighing around 30 g, at the times indicated by the arrows. △-----△ Catalase activity in the livers of mice with one injection only, ○——○ catalase activity in the livers of mice with three injections.

Fig. 1

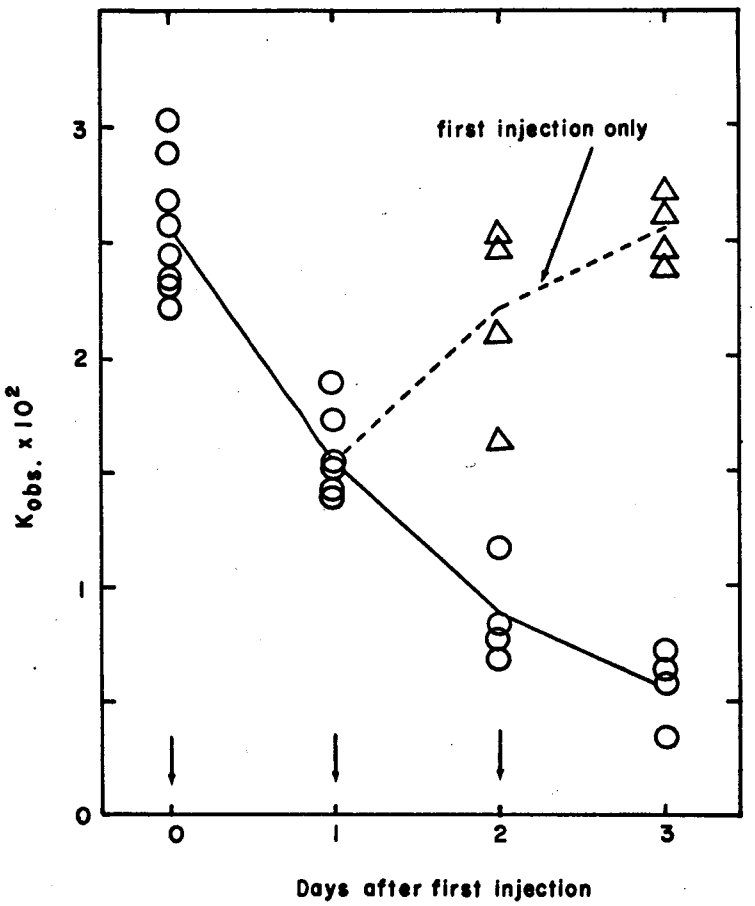

first injection only
$K_{obs.} \times 10^2$
0
1
2
3
0
1
2
3
Days after first injection

Fig. 2. Effect of injection of Rhodamine sarcoma nuclei and liver nuclei into mice on liver catalase activity

The arrows indicate the time when nuclei were injected. Other experimental conditions were the same as those for Fig. 1. ○——○ Catalase activity in the livers of mice injected with Rhodamine sarcoma nuclei (sarcoma nuclei), △----△ catalase activity in the livers of mice injected with liver nuclei (liver nuclei).

Fig. 2

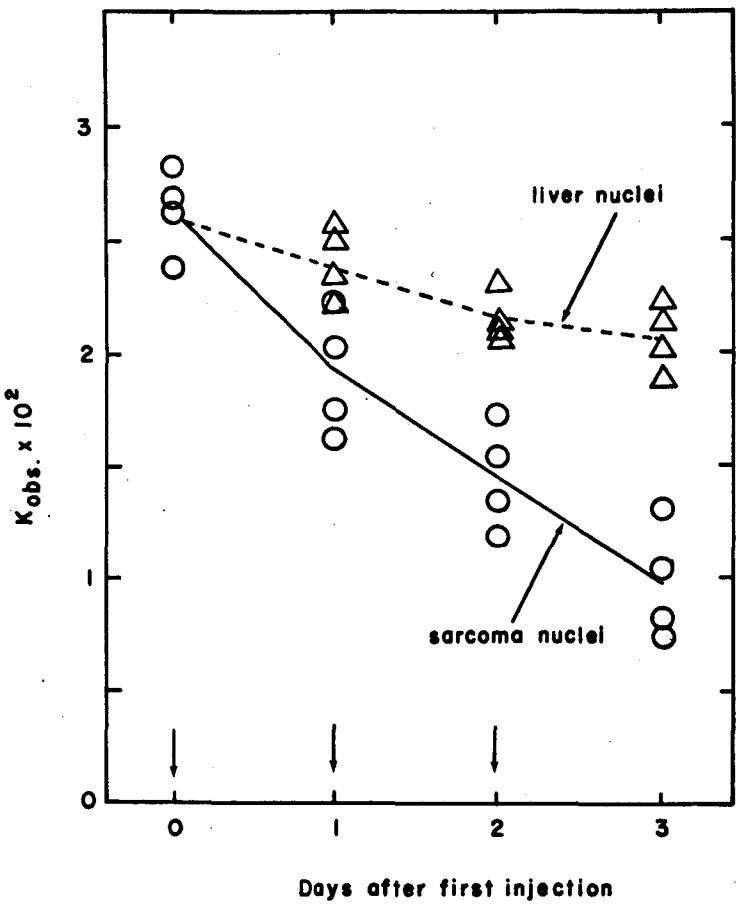

liver nuclei
sarcoma nuclei
$K_{obs.} \times 10^2$
Days after first injection
0
1
2
3

Fig. 3. Effect of injection of Rhodamine sarcoma nuclei into mice on catalase activities of particulate and soluble fractions from livers

Rhodamine sarcoma nuclei were injected into mice at the times indicated by the arrows. Livers from the mice were fractionated into particulate and soluble fractions. ○——○ Catalase activity of particulate fraction, △——△ catalase activity of soluble fraction.

Fig. 3

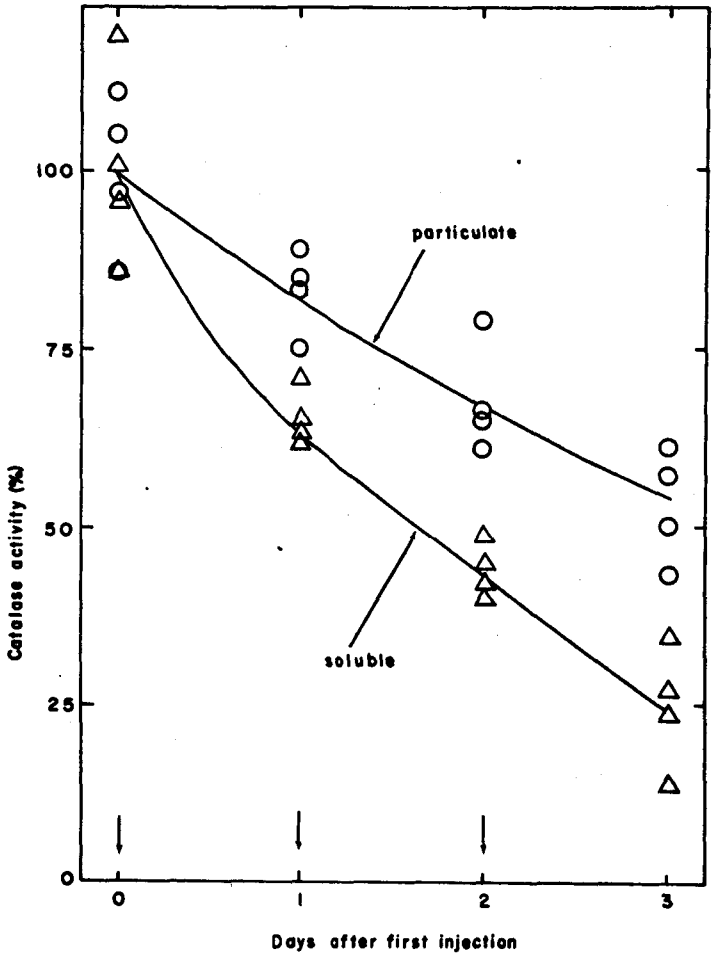

100
75
50
25
0
Catalase activity (%)
particulate
soluble
0
1
2
3
Days after first injection

Fig. 4. Immunotitration of catalase protein present in deoxycholate extracts from livers of normal mice and mice injected with Rhodamine sarcoma nuclei

○——○ With normal mice, ●——● with mice injected with Rhodamine sarcoma nuclei.

Fig. 4

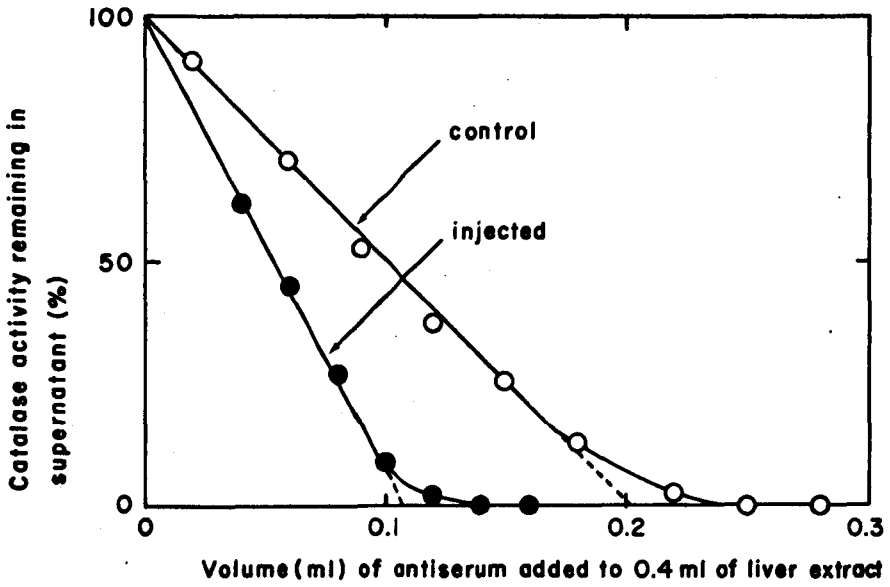

100
50
0
0
0.1
0.2
0.3
control
injected
Catalase activity remaining in supernatant (%)
Volume (ml) of antiserum added to 0.4 ml of liver extract

Fig. 5. Effect of injection of Rhodamine sarcoma nuclei into mice on amount and activity of catalase present in deoxycholate extracts from livers

Rhodamine sarcoma nuclei were injected at the time indicated by the arrows.

○——○ Catalase activity, △——△ amount of catalase protein.

Fig. 5

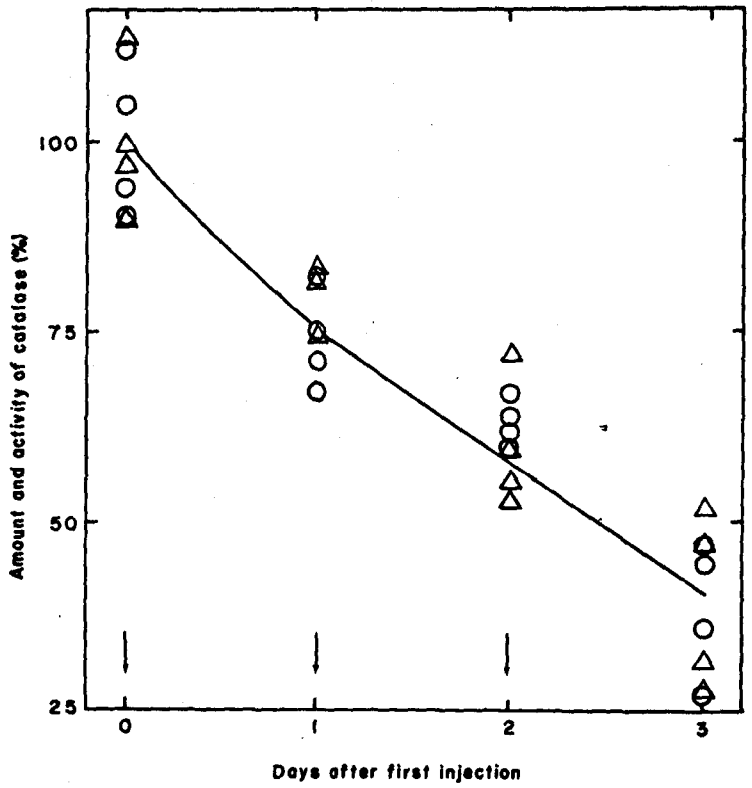

Amount and activity of catalase (%)
100
75
50
25
0
1
2
3
Days after first injection

Fig. 6. Effect of injection of Rhodamine sarcoma nuclei into mice previously injected with 3-amino-1,2,4-triazole on liver catalase activity

Aminotriazole and Rhodamine sarcoma nuclei were injected at the time indicated by the arrows.

○——○ Injection of aminotriazole alone, △——△ injection of aminotriazole followed by daily injection of Rhodamine sarcoma nuclei.

Fig. 6

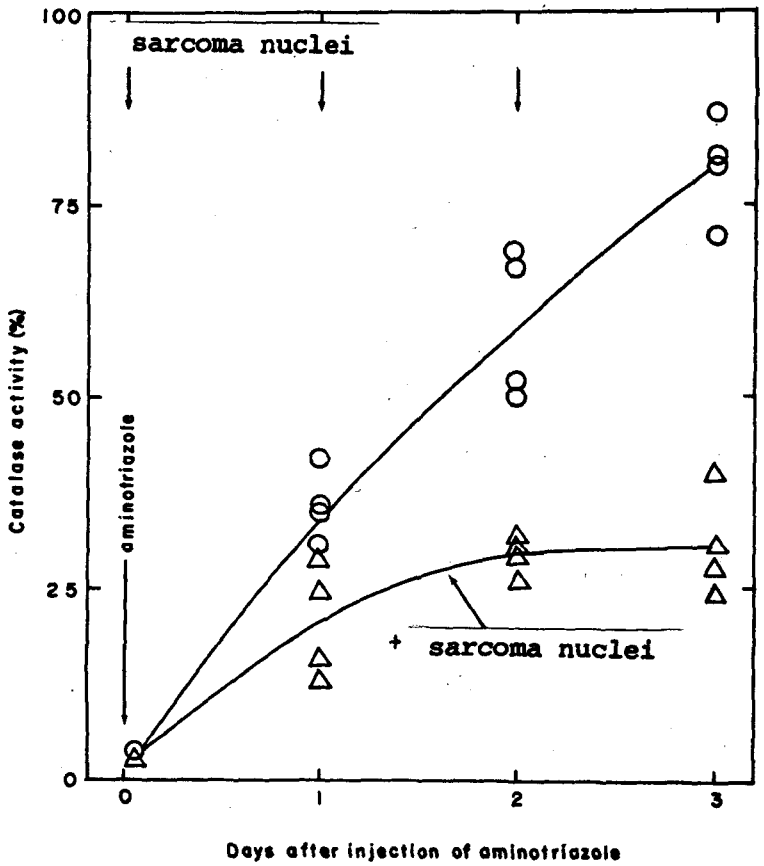

100
75
50
25
0
Catalase activity (%)
sarcoma nuclei
aminotriazole
+ sarcoma nuclei
0
1
2
3
Days after injection of aminotriazole

Fig. 7. Effect of injection of Rhodamine sarcoma nuclei into mice previously injected with 3-amino-1,2,4-triazole on catalase activities with soluble and particulate fractions from livers

The time at which aminotriazole and Rhodamine sarcoma nuclei were injected are indicated by the arrows.

○——○ and ●——● injection of aminotriazole alone, △——△ and ▲——▲ injection of aminotriazole followed by daily injections of Rhodamine sarcoma nuclei. Particulate, with particulate fractions; soluble, with soluble fractions.

Fig. 7

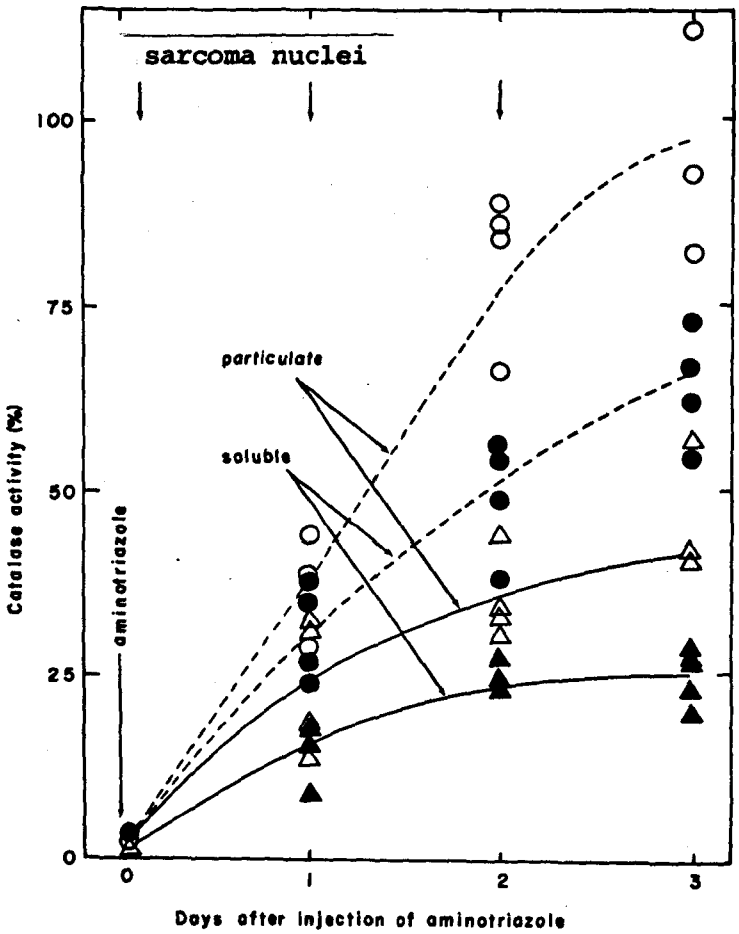

sarcoma nuclei
100
75
50
25
0
Catalase activity (%)
aminotriazole
particulate
soluble
0
1
2
3
Days after injection of aminotriazole

Fig. 8. Effect of injection of allylisopropylacetamide plus Rhodamine sarcoma nuclei into mice on liver catalase activity

The times when allylisopropylacetamide and Rhodamine sarcoma nuclei were injected, are indicated by the arrows. ○——○ Injection of allylisopropylacetamide, △——△ injection of allylisopropylacetamide plus Rhodamine sarcoma nuclei.

Fig. 8

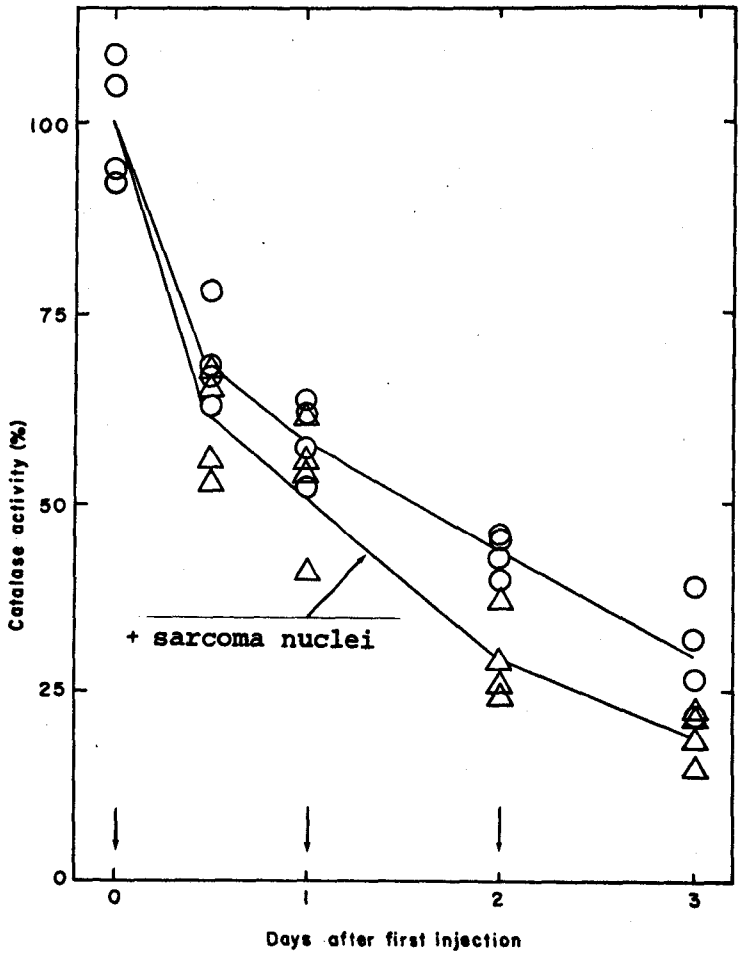

100
75
50
25
0
Catalase activity (%)
+ sarcoma nuclei
0
1
2
3
Days after first injection

Fig. 9. Effect of ammonium sulfate treatment on pI-isozyme pattern of pyruvate kinase from normal rat liver

A portion (1.5 ml) of the extract from normal rat liver was subjected to isoelectric separation with the use of Ampholine carrier ampholytes of pH from 3 to 10, the final concentration of which was 1% (w/v). After electrofocusing, the eluate was divided into 1-ml fractions. The pH and the activity of pyruvate kinase (U/ml) of each fraction were measured. In a parallel experiment, other portion of the same extract was subjected to the ammonium sulfate treatment by the procedure described in the text. The resulting precipitate was dissolved and desalted, and the solution thus obtained was subjected to isoelectric separation. Other experimental conditions were described in the text. ○——○ Without ammonium sulfate treatment, ●——● with ammonium sulfate treatment.

Fig. 9

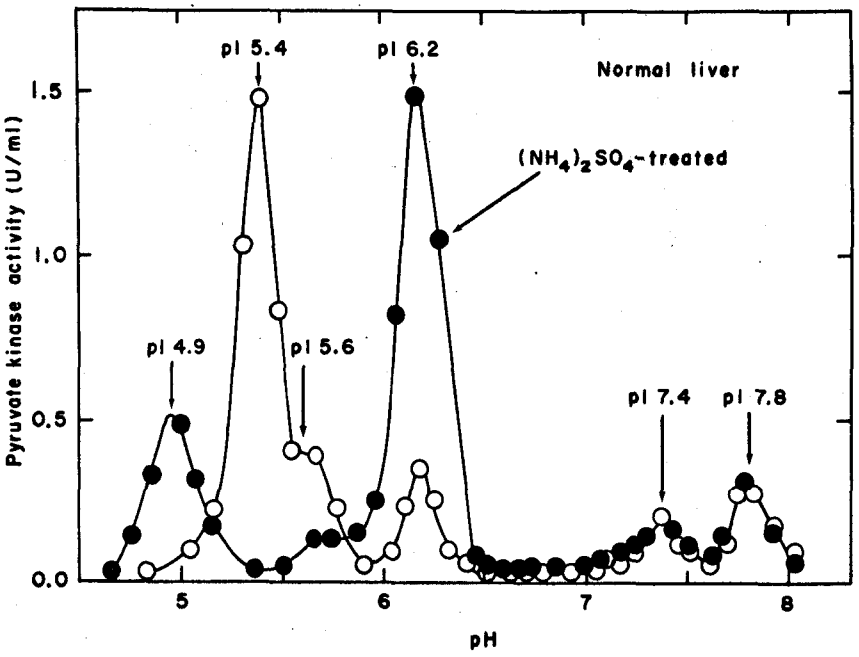

pI 5.4
pI 6.2
Normal liver
$(NH_4)_2SO_4$-treated
pI 4.9
pI 5.6
pI 7.4
pI 7.8
Pyruvate kinase activity (U/ml)
1.5
1.0
0.5
0.0
5
6
7
8
pH

Fig. 10. Repeated isoelectric separation of pI 5.4-isozyme and pI 6.2-isozyme obtained by isoelectric separation of extracts from normal rat liver

The fractions for pI 5.4-isozyme and those for pI 6.2-isozyme obtained in the experiment for Fig. 9 were subjected to isoelectric separation. Other experimental conditions were the same as those for Fig. 9. ○——○ Re-electrofocusing of liver pI 5.4-isozyme, △——△ re-electrofocusing of liver pI 6.2-isozyme. Re-electrofocusing of the pI 6.2-isozyme obtained from the isoelectric separation of extracts with and without ammonium sulfate treatment gave essentially the same result.

Fig. 10

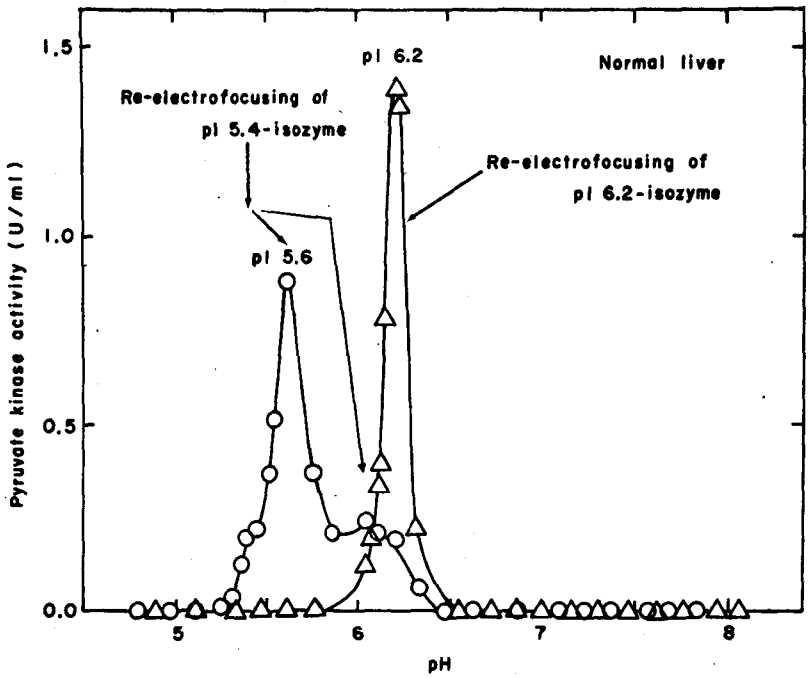

Pyruvate kinase activity (U/ml)
1.5
1.0
0.5
0.0
5
6
7
8
pH
pI 6.2
Normal liver
Re-electrofocusing of
pI 5.4-isozyme
Re-electrofocusing of
pI 6.2-isozyme
pI 5.6

Fig. 11. Effect of ammonium sulfate treatment on pI-isozyme pattern of pyruvate kinase from normal rat spleen

Experimental conditions were the same as those for Fig. 9, except that extracts from normal rat spleen were used. ○——○ Without ammonium sulfate treatment, △——△ with ammonium sulfate treatment.

Fig. 11

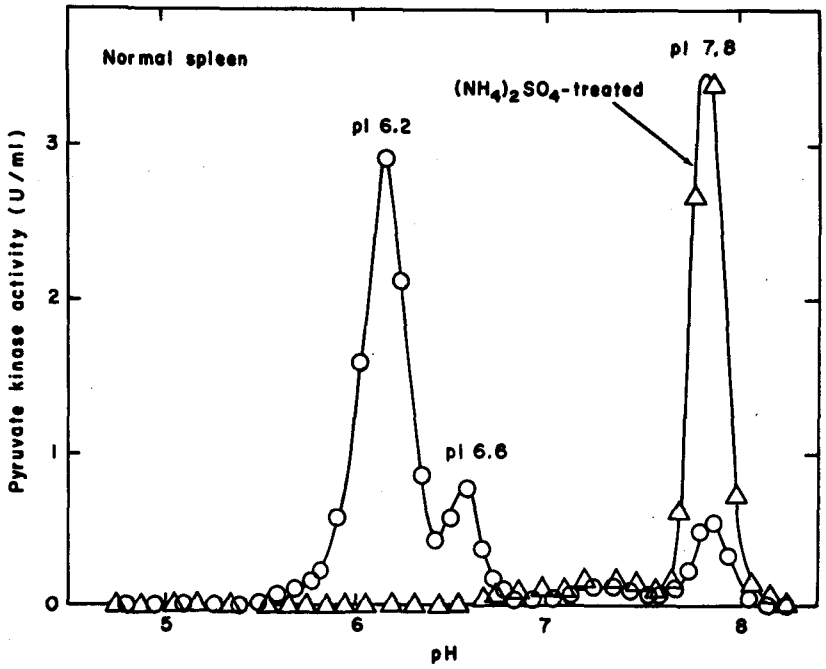

Normal spleen
pI 7.8
$(NH_4)_2SO_4$-treated
pI 6.2
pI 6.6
Pyruvate kinase activity (U/ml)
0
1
2
3
5
6
7
8
pH

Fig. 12. Repeated isoelectric separation of pI 6.2-isozyme and pI 7.8-isozyme obtained by isoelectric separation of extract from normal rat spleen

The fractions for pI 6.2-isozyme and those for pI 7.8-isozyme obtained in the experiment for Fig. 11 were subjected to isoelectric separation. Other experimental conditions were the same as those for Fig. 9.

○——○ Re-electrofocusing of spleen pI 6.2-isozyme, △——△ re-electrofocusing of spleen pI 7.8-isozyme.

Fig. 12

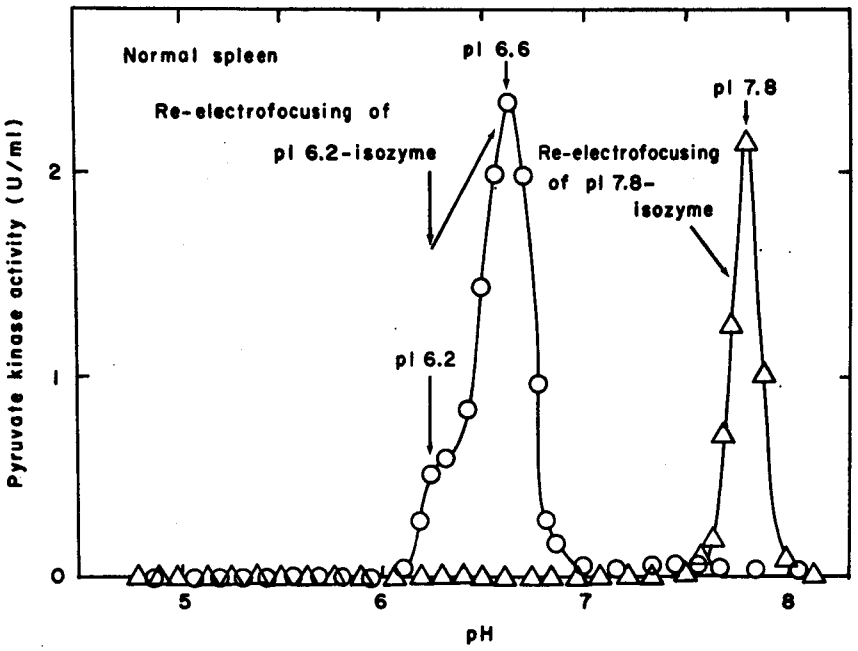

Normal spleen
pI 6.6
pI 7.8
Re-electrofocusing of
pI 6.2-isozyme
Re-electrofocusing
of pI 7.8-
isozyme
pI 6.2
Pyruvate kinase activity (U/ml)
0
1
2
5
6
7
8
pH

Fig. 13. Conversion of spleen pI 7.8-isozyme to pI 6.6- and pI 6.2-isozymes by binding with fructose 1,6-diphosphate

The experimental conditions were described in the text.

○———○ Re-electrofocusing of spleen pI 7.8-isozyme, △———△ re-electrofocusing of mixture of 5 ml of spleen pI 7.8-isozyme and 5 ml of 0.2 mM fructose 1,6-diphosphate, □———□ re-electrofocusing of spleen pI 7.8-isozyme in the electrofocusing column, in which all the solution contained 1 mM fructose 1,6-diphosphate.

Fig. 13

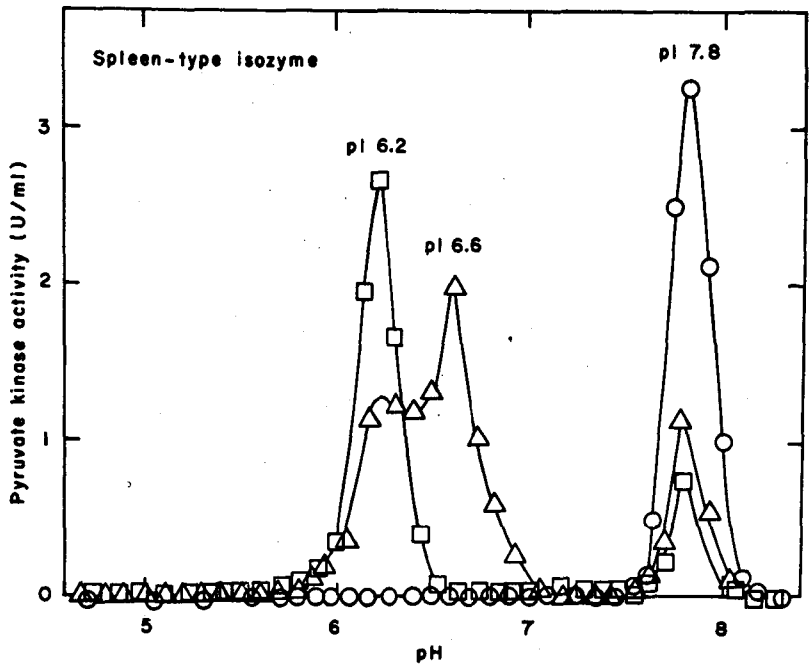

Spleen-type isozyme
pI 6.2
pI 6.6
pI 7.8
Pyruvate kinase activity (U/ml)
0
1
2
3
5
6
7
8
pH

Fig. 14. Effect of growth of Rhodamine sarcoma on pI-isozyme pattern of liver pyruvate kinase

Experimental conditions were the same as those for Fig. 9, except that extracts from Rhodamine sarcoma-bearing rat livers were used. ○——○ Without ammonium sulfate treatment, ●——● with ammonium sulfate treatment.

Fig. 14

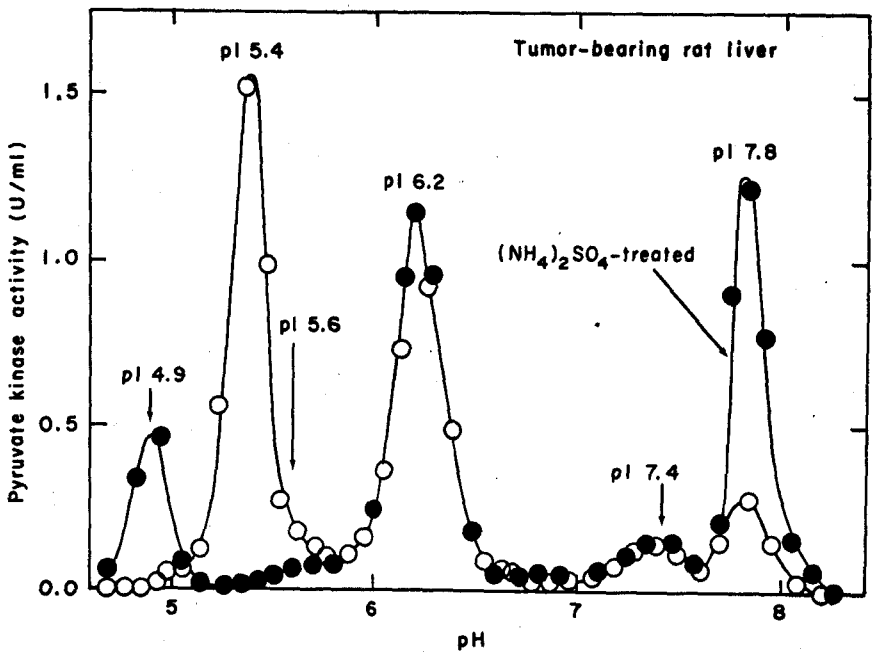

Tumor-bearing rat liver
pI 5.4
pI 6.2
pI 7.8
pI 5.6
pI 4.9
pI 7.4
$(NH_4)_2SO_4$-treated
Pyruvate kinase activity (U/ml)
1.5
1.0
0.5
0.0
5
6
7
8
pH

Fig. 15. *In vivo* liver catalase-depressing activities of chromatin and histone-free chromatin from Rhodamine sarcoma

Chromatin and histone-free chromatin were prepared from Rhodamine sarcoma. Both samples were suspended in 10 m*M* sodium phosphate buffer (pH 7.0) and separately injected into mice at a dose which contained 1.5 mg of DNA. The control mice were injected with 0.5 ml of the buffer.

Fig. 15

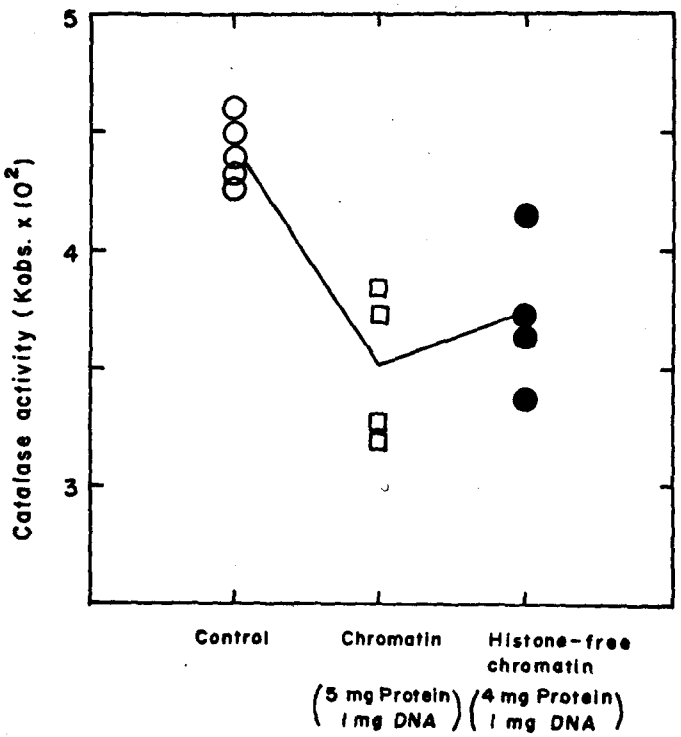

5
4
3
Catalase activity (Kobs. x (10²)
Control
Chromatin
(5 mg Protein / 1 mg DNA)
Histone-free chromatin
(4 mg Protein / 1 mg DNA)

Fig. 16. Isoelectric separation of acid extract from Rhodamine sarcoma chromatin

An acid extract from Rhodamine sarcoma chromatin (approximately 20 mg of protein) was subjected to isoelectric separation with the use of Ampholine carrier ampholytes.

Fig. 16

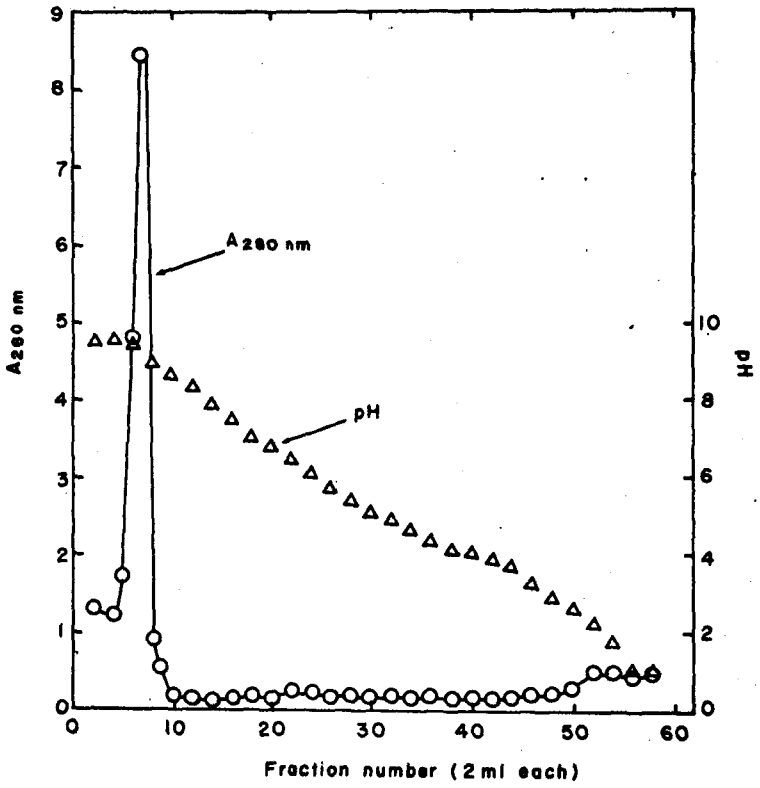

A 280 nm
pH
A280 nm
pH
Fraction number (2 ml each)
0
10
20
30
40
50
60
1
2
3
4
5
6
7
8
9

Fig. 17. *In vivo* liver catalase-depressing activities of histone from Rhodamine sarcoma, poly-L-lysine, and L-lysine

Histone from Rhodamine sarcoma, poly-L-lysine, and L-lysine were dissolved in 10 mM sodium phosphate buffer (pH 7.0), and separately injected into mice at the indicated doses. See Fig. 21 for F-2 (pI 4-7), which was a preparation of non-histone protein from Rhodamine sarcoma chromatin.

Fig. 17

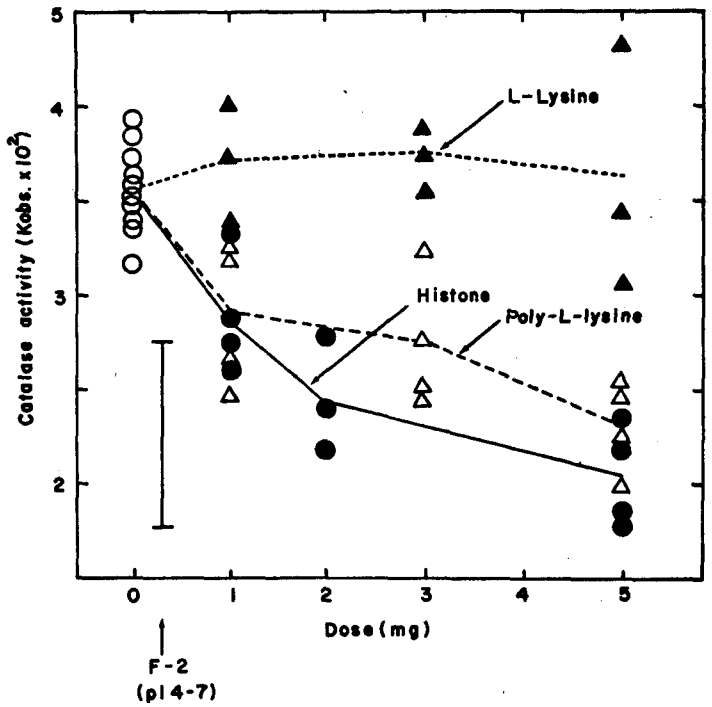

Catalase activity (Kobs. x 10²)
5
4
3
2
0
1
2
3
4
5
Dose (mg)
L-Lysine
Histone
Poly-L-lysine
F-2
(pI 4-7)

Fig. 18. Procedure for separation of components of Rhodamine sarcoma chromatin

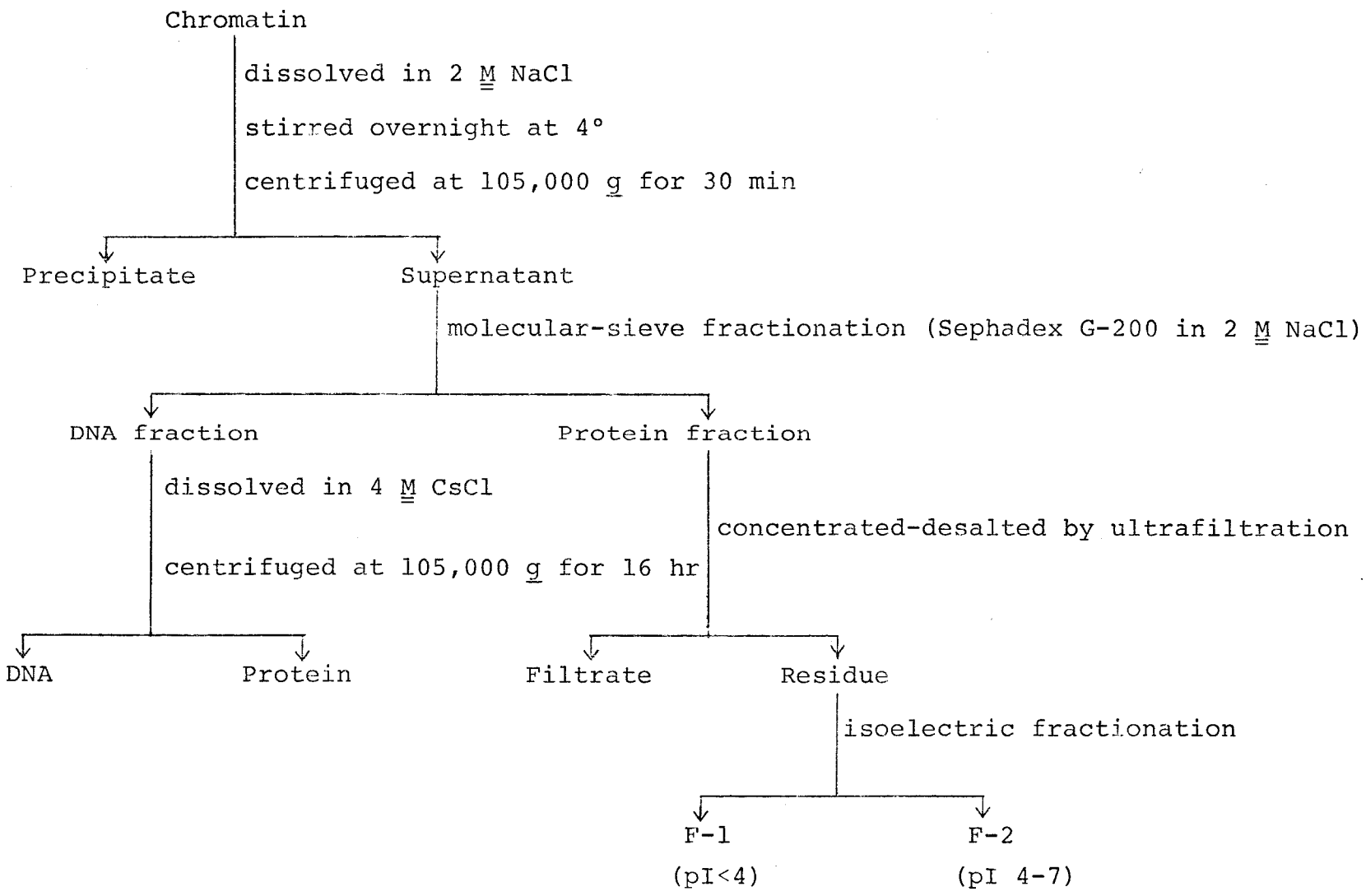


Fig. 19. Molecular-sieve fractionation of Rhodamine sarcoma chromatin by Sephadex G-200

A chromatin preparation from Rhodamine sarcoma, which contained approximately 150 mg of protein and approximately 50 mg of DNA, was dissolved in 50 ml of 2 M NaCl. The resulting solution was subjected to molecular-sieve fractionation on a Sephadex G-200 column (bed volume 1,900 ml), which had been equilibrated with 2 M NaCl. The charged column was developed with 2 M NaCl and the eluate was divided into 20-ml fractions. Each fraction was analyzed for DNA and protein contents.

Fig. 19

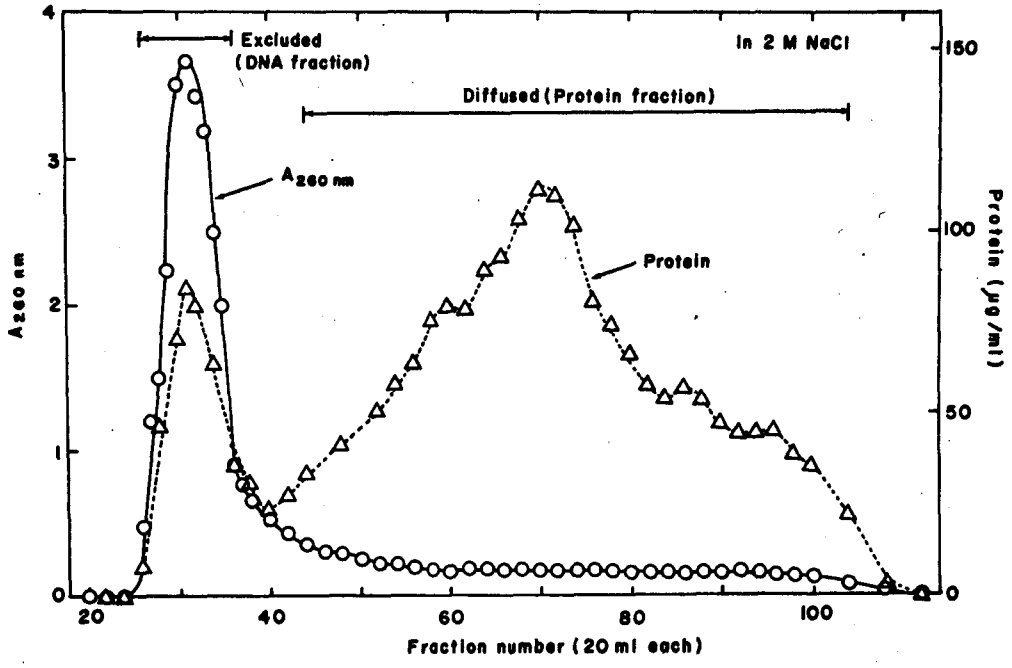

Excluded
(DNA fraction)
In 2 M NaCl
Diffused (Protein fraction)
$A_{260\,nm}$
Protein
$A_{260\,nm}$
Protein (μg/ml)
Fraction number (20 ml each)
0
1
2
3
4
20
40
60
80
100
0
50
100
150

Fig. 20. Isoelectric separation of chromosomal protein from Rhodamine sarcoma chromatin

Chromosomal protein of Rhodamine sarcoma was separated from DNA by molecular-sieve fractionation as shown in Fig. 19. The protein fractions were then concentrated-desalted by ultrafiltration. The resulting residue, which was mostly composed of non-histone protein and free of histone, was subjected to isoelectric separation. The content of the charged protein was 10 mg.

Fig. 20

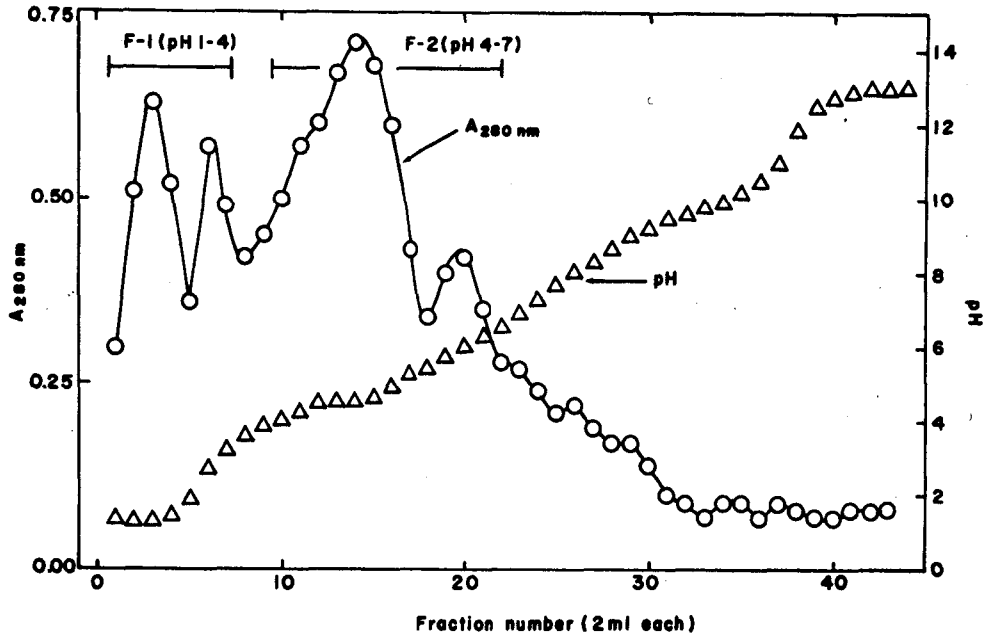

F-1 (pH 1-4)
F-2 (pH 4-7)
$A_{280\,nm}$
pH
0.75
0.50
0.25
0.00
$A_{280\,nm}$
14
12
10
8
6
4
2
0
pH
0
10
20
30
40
Fraction number (2ml each)

Fig. 21. *In vivo* liver catalase-depressing activities of DNA and two non-histone protein fractions from Rhodamine sarcoma chromatin

Protein-free DNA preparation was obtained from the chromatin of Rhodamine sarcoma by the procedure shown in Fig. 18. The DNA preparation (DNA) and the two non-histone protein fractions (F-1 and F-2; see Fig. 20) were subjected to assay of *in vivo* liver catalase-depressing activity at the doses indicated.

Fig. 21

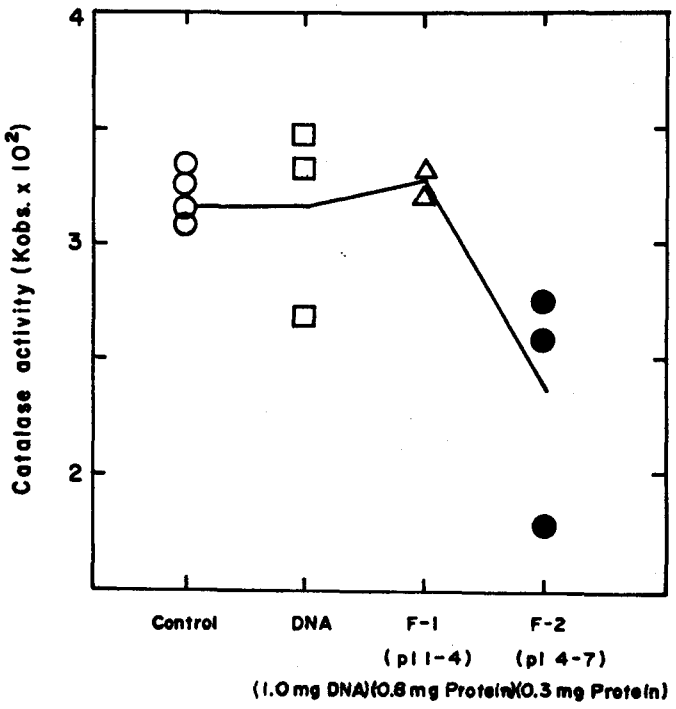

Catalase activity (Kobs. x 10²)
4
3
2
Control
DNA
F-1
(pI 1-4)
(1.0 mg DNA)
(0.8 mg Protein)
F-2
(pI 4-7)
(0.3 mg Protein)

Fig. 22. Absorbance spectra of chromatin, DNA and non-histone protein preparations from Rhodamine sarcoma

a) chromatin, b) DNA, c) non-histone protein

Fig. 22-a

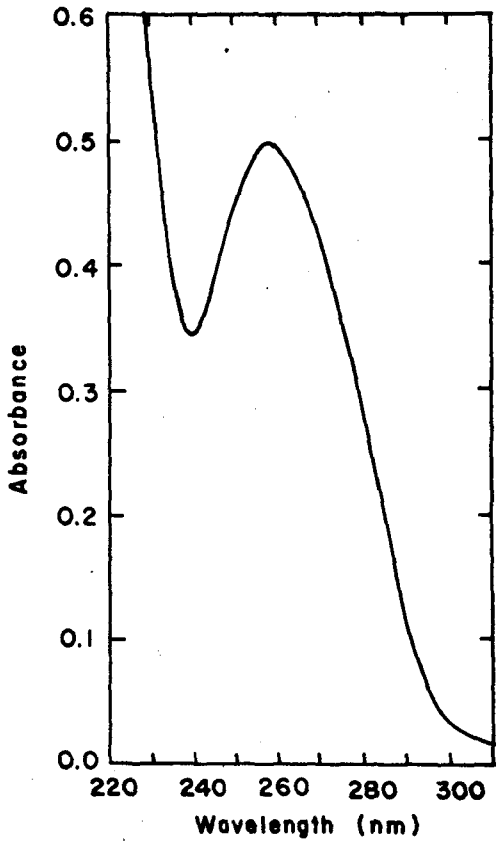

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
220
240
260
280
300
Wavelength (nm)

Fig. 22-b

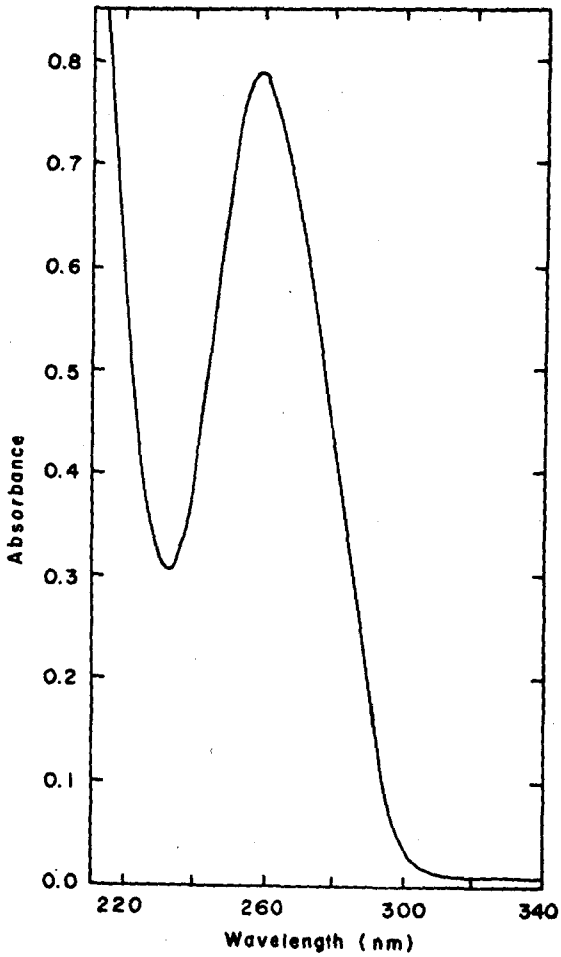

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
220
260
300
340
Wavelength (nm)

Fig. 22-C

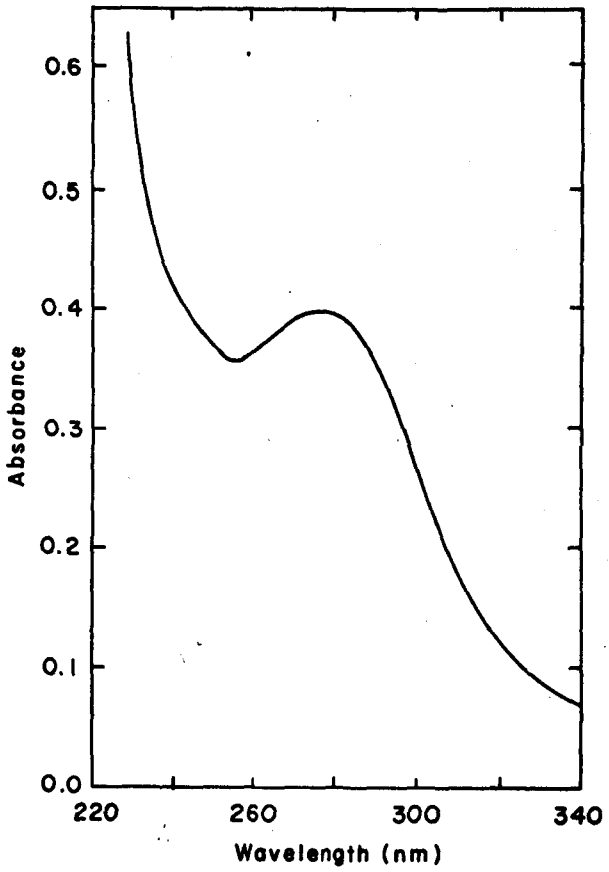


Fig. 23. Degradation of chromatin from Rhodamine sarcoma by endogeneous protease and deoxyribonuclease

One ml of the standard reaction mixture was composed of Rhodamine sarcoma chromatin (1.0 mg of protein and 0.35 mg of DNA) and 10 m$\underline{\underline{M}}$ Tris-HCl buffer (pH 8.0). The reaction mixtures with and without 10 m$\underline{\underline{M}}$ sodium citrate were incubated at 37°, and the reactions were stopped by adding 1 ml of 10% trichloric acid (TCA) at the indicated times, followed by centrifugation at 3,000 $\underline{g}$ for 10 min. The resulting supernatants were analyzed for protein and DNA contents.

○——○ Protein content in the standard reaction mixture, ●——● protein content in the reaction mixture containing 10 m$\underline{\underline{M}}$ Na-citrate, △——△ DNA content in the standard reaction mixture, ▲——▲ DNA content in the reaction mixture containing 10 m$\underline{\underline{M}}$ Na-citrate.

Fig. 23

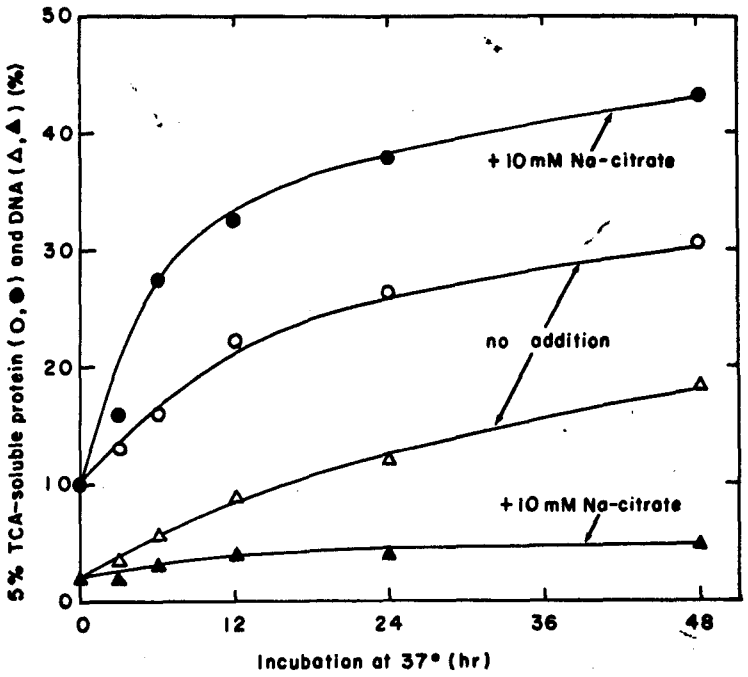

5% TCA-soluble protein (O, ●) and DNA (Δ, ▲) (%)
50
40
30
20
10
0
+ 10 mM Na-citrate
no addition
+ 10 mM Na-citrate
0
12
24
36
48
Incubation at 37° (hr)

Fig. 24. Effect of various treatments on proteolytic activity of chromatin from Rhodamine sarcoma

The reaction mixtures containing Rhodamine sarcoma chromatin were incubated under various conditions, and proteolytic activity was analyzed by the method described in Fig. 23. ●——● No addition, △——△ +10 m$\underline{\underline{M}}$ $NaHSO_3$, □——□ +20 μg/ml soybean trypsin inhibitor, ○——○ incubation at 0°, ▲——▲ chromatin previously heated at 90° for 3 min.

Fig. 24

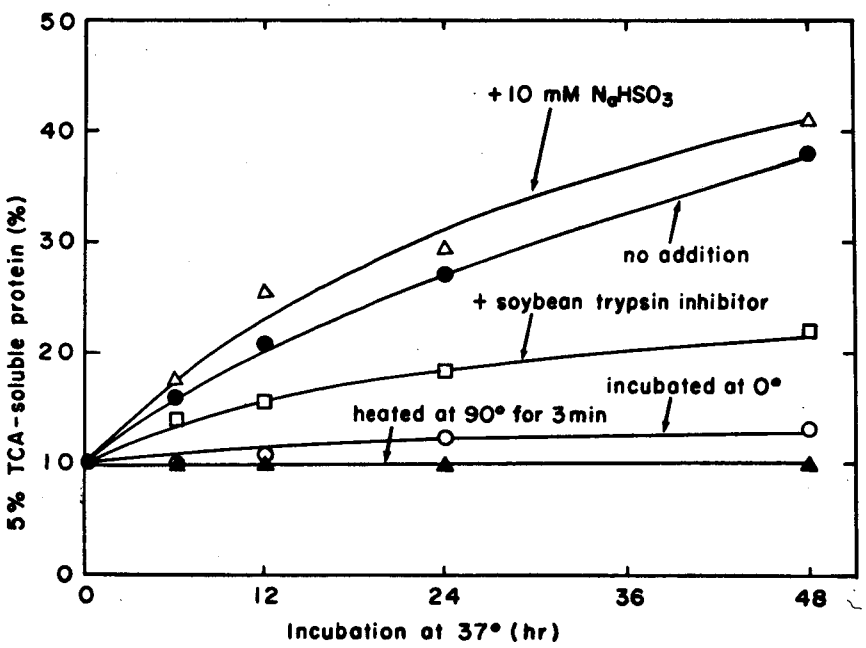

+10 mM N_aHSO_3
no addition
+ soybean trypsin inhibitor
incubated at 0°
heated at 90° for 3min
5% TCA-soluble protein (%)
Incubation at 37° (hr)
0
10
20
30
40
50
0
12
24
36
48

Fig. 25. Procedure for isolation of *in vivo* liver catalase-depressing substance from Rhodamine sarcoma nuclei

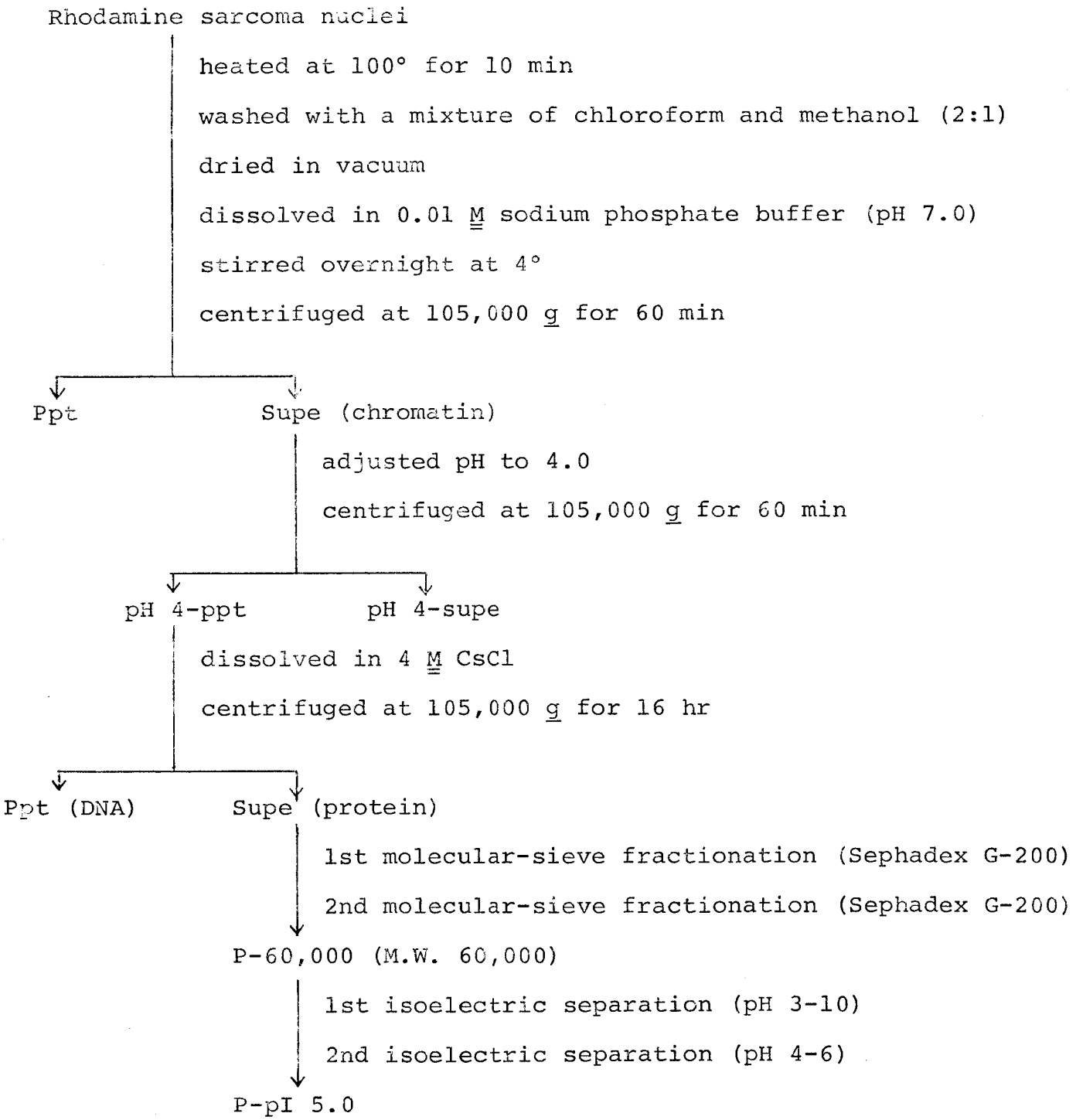


Fig. 26. Fractionation of pH 4-ppt into protein and DNA by centrifugation in the presence of 4 M CsCl

pH 4-Ppt fraction of chromatin was prepared from Rhodamine sarcoma nuclei by the procedure described in Fig. 25, and dissolved in 0.01 M sodium phosphate buffer (pH 7.0) containing 4 M CsCl. The solution (4.8 mg protein/ml) was centrifuged at 105,000 g for 16 hr. Tube content was divided into 5 fractions (2.2 ml each), and each fraction was analyzed for DNA and protein contents. ○——○ Protein, △——△ DNA.

Fig. 26

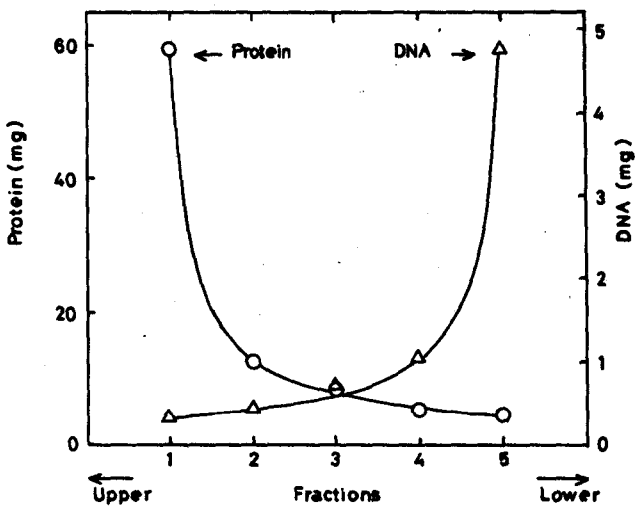

Protein
DNA
Protein (mg)
DNA (mg)
60
40
20
0
5
4
3
2
1
0
1
2
3
4
5
Upper
Fractions
Lower

Fig. 27. *In vivo* liver catalase-depressing activities of DNA and protein fractions obtained from pH 4-ppt

DNA and protein fractions were prepared from pH 4-ppt by the procedure shown in Fig. 26. They were subjected to assay of *in vivo* liver catalase-depressing activity at the indicated doses.

Fig. 27

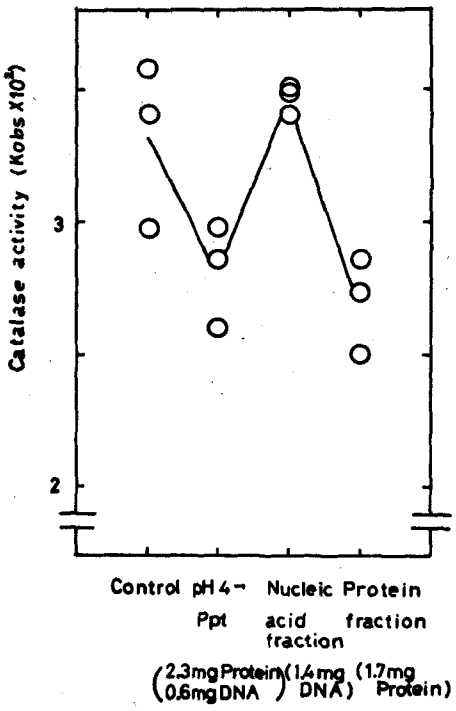

Catalase activity (Kobs X10³)
3
2
Control
pH 4 Ppt
Nucleic acid fraction
Protein fraction
(2.3mg Protein 0.6mg DNA)
(1.4mg DNA)
(1.7mg Protein)

Fig. 28. Molecular-sieve fractionation on Sephadex G-200 column of protein fraction from pH 4-ppt

A solution of protein fraction (280 mg) in 15 ml of 0.01 M sodium phosphate buffer (pH 7.0) was subjected to molecular-sieve fractionation on a Sephadex G-200 column (bed volume, 500ml), which had been equilibrated with the same buffer (1st molecular-sieve fractionation). The eluate was divided into 5-ml aliquots. The aliquots from fraction 53 to 63 were mixed, concentrated by lyophilization and then subjected to 2nd molecular-sieve fractionation in the same manner as that for the 1st molecular-sieve fractionation. Essentially, the same elution pattern was obtained when 0.1 M ammonium formate buffer (pH 7.0)[11] was used instead of the sodium phosphate buffer. ○——○ 1st molecular-sieve fractionation, ●——● 2nd molecular-sieve fractionation.

Fig 28

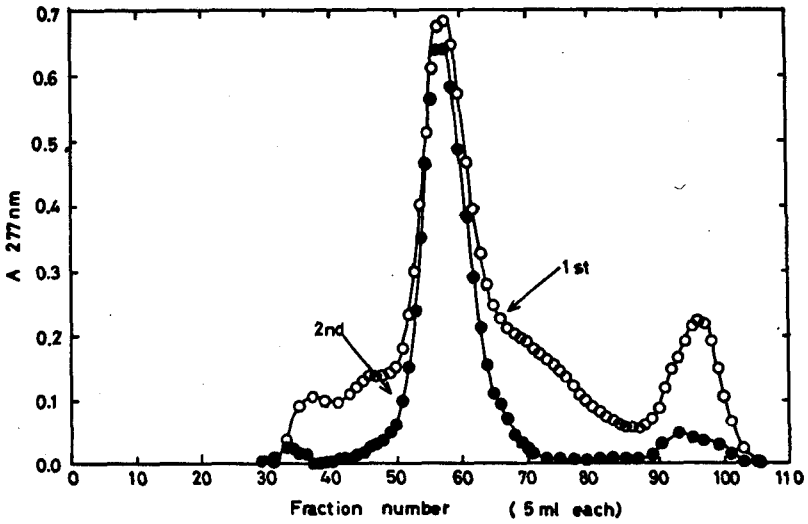

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A 277nm
0
10
20
30
40
50
60
70
80
90
100
110
Fraction number (5 ml each)
1st
2nd

Fig. 29. *In vivo* liver catalase-depressing activities of fractions obtained by molecular-sieve fractionation and isoelectric separation

Fractions of P-60,000 and P-pI 5.0 were prepared by the procedures shown in Fig. 28 and Fig. 31, respectively. They were subjected to assay of *in vivo* liver catalase-depressing activity at the indicated doses.

Fig. 29

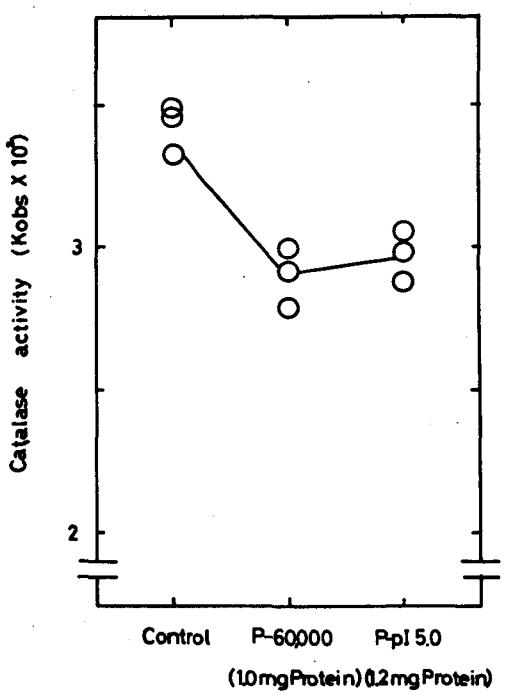

Catalase activity (Kobs X 10⁷)
3
2
Control
P-60,000
P-pI 5.0
(1.0mgProtein)
(0.2mgProtein)

Fig. 30. Molecular-sieve fractionation on Sephadex G-100 column of P-60,000

The fractions from 53 to 63 in 2nd molecular-sieve fractionation (Fig. 28) were mixed, concentrated by lyophilization and then subjected to 3rd molecular-sieve fractionation on a Sephadex G-100 column. The other experimental conditions were the same as those described in Fig. 28.

Fig. 30

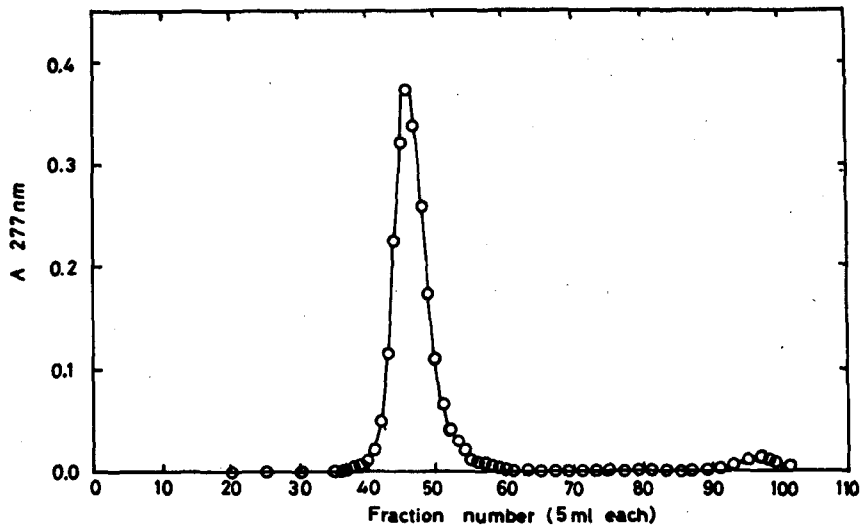

0.4
0.3
0.2
0.1
0.0
A 277nm
0
10
20
30
40
50
60
70
80
90
100
110
Fraction number (5ml each)

Fig. 31. Isoelectric separation of P-60,000

The same sample obtained by 2nd molecular-sieve fractionation on a Sephadex G-200 column as in Fig. 30 was subjected to isoelectric separation with the use of Ampholine carrier ampholytes of pH from 3 to 10.

Fig. 31

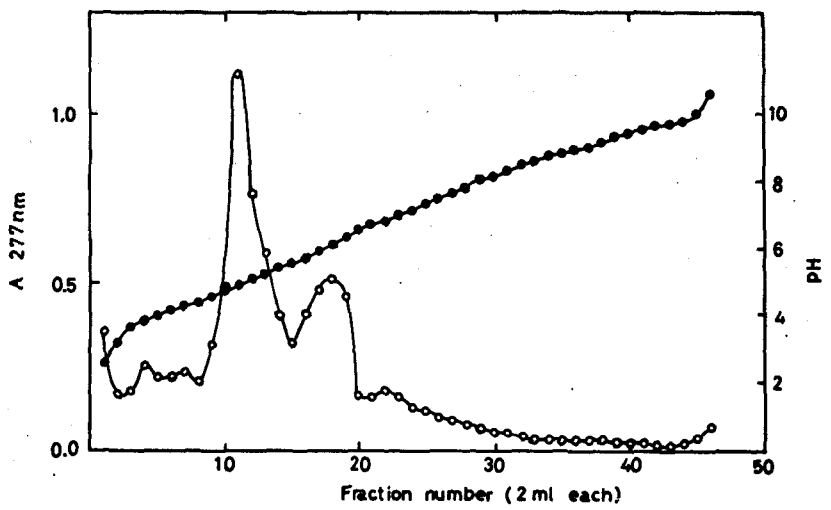
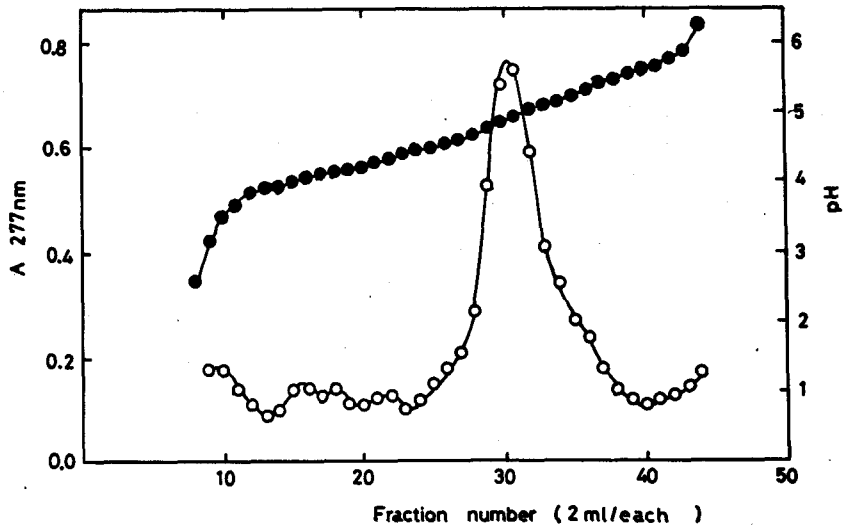

A 277nm
1.0
0.5
0.0
10
20
30
40
50
Fraction number (2 ml each)
pH
10
8
6
4
2

Fig. 32. Isoelectric separation of P-pI 5.0

The fractions from 29 to 32 in 1st isoelectric separation (Fig. 31) were mixed, desalted by dialysis, and subjected to 2nd isoelectric separation with the use of Ampholine carrier ampholytes of pH 4 to 6.

Fig. 32


0.8
0.6
0.4
0.2
0.0
A 277nm
6
5
4
3
2
1
pH
10
20
30
40
50
Fraction number (2 ml/each)