BIOCHEMICAL STUDIES ON CACHEXY DUE TO CANCER. V.* A HYPOALBUMINEMIC SUBSTANCE FROM EHRlich ASCITES CARCINOMA CELLS

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A hypoalbuminemic substance was purified from mouse Ehrlich ascites carcinoma cells by ammonium sulfate fractionation, adsorption on DEAE-cellulose, gel filtration on Sephadex G-200, treatment with butanol, ultracentrifugation, and gel filtration on Sephadex G-75. Electrophoresis on polyacrylamide gel containing sodium dodecylsulfate gave a single band of protein which corresponded to a molecular weight of 53000. This band was also detected by staining with methylene blue. Significant decreases in serum albumin, serum total protein, and leukocyte count were observed when it was given to mice by intraperitoneal injection. It stimulated the phagocytic activities of the reticuloendothelial system of mice. Of these effects, only the leukocyte-lowering effect disappeared when it was administered to adrenalectomized mice. Hydrocortisone produced a marked decrease in the leukocyte count but no significant change in the serum albumin and total protein when given to normal mice over a period of six days. The vascular permeability-increasing activity concentrated into the fraction unadsorbed on DEAE-cellulose and distinctly separated from the hypoalbuminemic activity. The hypoalbuminemic substance was composed of 28.9% of DNA, 24.9% of hexose, 24.6% of RNA, 14.6% of amino acid residues, and 1% of hexosamine. The lipid content was negligible. The hypoalbuminemic activity decreased considerably at pH 2 or 11 and almost completely disappeared by heating at 60°. This activity was decreased markedly by the partial digestion with Pronase, trypsin, or DNase, whereas the treatment with RNase or the butanol extraction exerted no influence on the activity.

Keywords—hypoalbuminemic substance; Ehrlich ascites carcinoma cells; hypoalbuminemic activity; hypoproteinemic activity; leukocyte-lowering activity; hypoalbuminemia; leukopenia; cachexy

INTRODUCTION

Hypoalbuminemia is one of the characteristic biochemical changes which generally accompany cachexy in cancer patients and in tumor-bearing animals. Little has, however, been reported so far about the mechanism by which the hypoalbuminemia is produced. The hypoalbuminemic substance (F-3) which was extracted from mouse Ehrlich ascites carcinoma¹⁾ may be one of the factors responsible for the hypoalbuminemia in mice

bearing the tumor. This substance also showed marked vascular permeability-increasing, leukocyte-lowering, and reticuloendothelial system-stimulating effects.^{2a,b)} It is, however, obscure at the present stage whether or not these effects are separately produced by two or more active components.

The purpose of the present work is to clarify this problem by the purification and characterization of the hypoalbuminemic substance. A short com-

* Part IV: H.Ueki, S.Tsunemi, and Y.Kubota, *Yakugaku Zasshi*, 95, 574 (1975).

munication of this work has been published.³⁾

MATERIALS AND METHODS

Materials—Cellulose acetate paper (Separax) was purchased from Joko Sangyo Co., Tokyo. Pelikan ink was a product of Günther Wagner, Germany. Trypan Blue and Ponceau 3R were purchased from Chroma-Gesellschaft Schmidt Co., Germany. Bovine plasma albumin (Fraction V of Cohn) was obtained from Armour Pharmaceutical Co., U.S.A. Mouse albumin (Fraction V Powder) was obtained from Miles Laboratories, Illinois. Ovalbumin and Silica gel G were purchased from Boehringer-Mannheim, Germany. Pronase (*Streptomyces griseus* protease, Pronase E, 70000 PUK/g) was a gift from Kaken Chemical Co., Tokyo. Trypsin, soybean trypsin inhibitor, and DNase (from beef pancreas) were obtained from Sigma Chemical Co., St. Louis. RNase (from beef pancreas) and sodium dodecylsulfate were purchased from Tokyo-kasei Kogyo, Tokyo. Tosyl-L-arginine methyl ester was a product of the Protein Research Foundation, Osaka. L-(1-Tosyl-amido-2-phenyl)ethyl chloromethyl ketone (TPCK) and sodium cholate were obtained from Nakarai Chemicals Co., Kyoto. Hydrocortisone (Saxizon, hydrocortisone sodium succinate) was purchased from Nikken Chemicals Co., Tokyo. Sephadex (Pharmacia) and DEAE-cellulose (Serva) were purchased from Seikagaku Kogyo Tokyo.

Animals and Tumor Cells—Mice, dd-K males weighing about 25 g, were obtained from Kuroda Animal Farm in Kumamoto. Ehrlich ascites carcinoma cells (2×10^6 /mouse) were inoculated intraperitoneally into mice. After 10 days of growth, the cells were collected from the mice and washed six times with cold physiological saline by centrifugation for five min at 1000 rpm (CD-50 centrifuge, Tominaga). The packed cells were prepared by centrifugation of the collected cells for 20 min at 3000 rpm.

Adrenalectomy—Adrenalectomy was carried out according to the method of Firor *et al.*⁴⁾ Sham-operation involved all operation except for lifting out the adrenals. All the adrenalectomized mice showed a gradual decrease in body weight and died

by the ninth day.

Assays—Samples dissolved in physiological saline were given to mice by a single intraperitoneal injection. After 24 hr, blood samples (0.3 ml) were taken from the tail vein and centrifuged for 10 min at 3000 rpm. Serum total protein was determined by the method of Folin-Lowry.⁵⁾ The ratio of albumin was calculated from the density of the electrophorogram of the serum. An aliquot of 8 μ l of the serum was applied to a cellulose acetate paper (2 $\times$ 6 cm) wetted with 0.05 M veronal buffer, pH 8.6. Electrophoresis was carried out with the same buffer for 45 min at 20° with a constant current of 6 mA/cm in width. The paper was stained with 0.8% Ponceau 3R containing 6% trichloroacetic acid and destained with 1% acetic acid. After drying in the air, the stained paper was immersed in liquid paraffin, and the density of the electrophorogram was measured at 524 nm with a densitometer (Densitorol DM-6, Toyo Kagaku, Osaka). Serum albumin level was calculated from the ratio of albumin to the serum total protein. The hypoalbuminemic activity is expressed as the percentage of a decrease in serum albumin as follows:

$$= \frac{\text{the mean value of treated group} - \text{that of control}}{\text{that of control}} \times 100$$

The leukocyte count was calculated simultaneously according to the routine method.^{2b)}

The vascular permeability-increasing action was determined in mice by the method based on the leakage of circulating Trypan Blue.^{2a, 6a, b)} An aliquot of 0.2 ml of 1% Trypan Blue solution was injected into the tail veins of mice and, after 10 min, samples dissolved in a constant volume of 0.05 ml of physiological saline were injected into the skin of the abdomen which had been depilated one day before. The animals were sacrificed 30 min after the injection of the dye, and the standard area of the skin was removed. The dye was extracted from the skin with 25% hot pyridine after extraction of lipid with ethanol. The concentration of the dye was determined colorimetrically at 600 nm.

The phagocytic activity of the reticuloendothelial system of mice was determined by measurement of the clearance of colloidal car-

bon.^{2b,7)} Pelikan ink was diluted four times with 1% gelatin, and 0.2 ml of this carbon solution was injected into the tail veins of mice 24 hr after intraperitoneal injection of samples. The phagocytic index, K , was calculated from the following equation;

$$K = \frac{\log A_{4\text{min}} - \log A_{20\text{min}}}{16}$$

where $A_{4\text{min}}$ and $A_{20\text{min}}$ represent the carbon concentration in the blood at 4 and 20 min, respectively.

Esterolytic activity⁸⁾ was determined in 0.1 M Tris-HCl buffer, pH 8.0, with tosyl-L-arginine methyl ester as substrate, and its unit was expressed as μmol of tosyl-L-arginine methyl ester hydrolyzed per min per mg of protein. Caseinolytic activity⁹⁾ was determined in 0.1 M phosphate buffer, pH 7.6, with 1% casein, and its unit was expressed as μmol of tyrosine released per min per mg of protein.

Extraction and Purification of Active Principle—The extraction and purification of the hypoalbuminemic substance were carried out as described in a previous paper.³⁾ Briefly, the packed cells were homogenized with physiological saline, extracted for 4 hr, and centrifuged for 30 min at $16000\times g$. To the supernatant (F-I)* obtained, an equal volume of a saturated solution of ammonium sulfate was added at pH 7.0. After centrifugation and dialysis, DEAE-cellulose was added to the supernatant (F-II) in 0.01 M Tris-HCl buffer, pH 7.6. The mixture was stirred for 2 hr and filtered through a sintered glass filter. As described later, the unadsorbed fraction showed a marked vascular permeability-increasing action but not a hypoalbuminemic activity. The fraction (F-III) possessing a hypoalbuminemic activity was eluted from the residue with 1 M NaCl, pH 4.0. F-III was subjected to gel filtration on Sephadex G-200 ($1.5\times 34\text{ cm}$) with 1 M NaCl as eluant. The major peak (F-IV) showed a hypoalbuminemic activity. F-IV was subjected to rechromatography on Sephadex G-200 under the same conditions. The

main peak (F-V) showed a hypoalbuminemic activity. After extraction of the lipid of F-V with butanol,¹⁰⁾ an equal volume of 20% sodium cholate was added, and the mixture was centrifuged for 30 min at $110000\times g$ in a Hitachi 65P ultracentrifuge. The supernatant (F-VI) was applied to a Sephadex G-75 column ($1.1\times 33\text{ cm}$) and eluted with 0.9% NaCl, pH 7.2. The main peak (F-VII) showed a hypoalbuminemic activity.

Electrophoresis on Polyacrylamide Gel Containing Sodium Dodecylsulfate—A mixture of 20 μl each of protein solution (0.1 to 0.5%) and 0.02 M phosphate buffer, pH 7.2, containing 50% glycerol, 10% 2-mercaptoethanol, and 2% sodium dodecylsulfate was incubated for 2 hr at 50° . To this, 10 μl of 0.05% bromophenol blue was added as a marker, and the mixture was applied to a $0.65\times 8\text{ cm}$ gel tube. Electrophoresis was carried out in 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate with a DISC MODEL 2 apparatus of Joko Sangyo Co., Tokyo, essentially by the method of Weber and Osborn.¹¹⁾ The gels were stained with 0.5% Amidoschwarz 10B in 20% acetic acid for detection of protein and with 2% methylene blue in 1 M acetic acid for nucleic acid.

Amino Acid Analysis—The sample (1 mg) was hydrolyzed with 0.5 ml of 6 N HCl under vacuum in a sealed pyrex glass tube for 24 hr at 110° . After removal of HCl under reduced pressure, the hydrolysate was dissolved in 2 ml of 0.2 M citrate buffer, pH 2.2, and the amino acid contents were determined with a Hitachi KLA-5 amino acid analyzer.¹²⁾ Hexosamine was estimated simultaneously. The content of tryptophan was determined on a separate, unhydrolyzed sample by the ultraviolet spectrophotometric method.¹³⁾

Carbohydrate Analysis—For the qualitative test,¹⁴⁾ the sample (1 mg) was hydrolyzed with 0.5 ml of 3 N HCl under vacuum in a sealed pyrex glass tube for 6 hr at 100° . After removal of HCl under reduced pressure, the hydrolysate was dissolved in 0.01 ml of deionized water. The solution was applied to a thin layer plate of Silica gel G, 0.25 mm in thickness and chromatographed with pro-

* F-I and -II were called F-1 and -2, respectively, in a previous paper.¹⁾

panol–water (7:1, v/v). After air-drying, the plate was sprayed with a 2.5% solution of aniline hydrogen phthalate and heated at 105° for 10 min. The content of hexose was determined by the anthrone method.¹⁵⁾

Nucleic Acid Analysis—The nucleic acid was separated according to the Schmidt-Thannhauser-Schneider procedure.^{16a, b)} The dried sample (0.5 mg) was dissolved in 1 ml of deionized water. To this, 2.5 ml of cold 10% trichloroacetic acid was added with stirring in an ice-cooled bath, and the mixture was centrifuged twice for 10 min at 1000×*g*. The precipitate obtained was suspended in 1 ml of deionized water. To this suspension, 4 ml of 95% ethanol was added and centrifuged. To remove lipid, the resulting precipitate was extracted three times with 5 ml of ethanol–ether (3:1, v/v) with heating in a boiling-bath for 30 min. The residue was extracted with 5 ml of 5% trichloroacetic acid for 5 min at 90° and centrifuged after cooling to obtain supernatant. The residue was extracted again with 2.5 ml of 5% trichloroacetic acid for 5 min without heating. The combined supernatant served as the nucleic acid fraction. The contents of DNA and RNA in this fraction were determined with diphenylamine¹⁷⁾ and orcinol,¹⁸⁾ respectively.

Lipid Analysis—The dried sample (3 mg) was suspended in 3 ml of chloroform–methanol (1:1, v/v), stirred for 20 hr at 4°, and centrifuged for 20 min at 1000×*g*. The residue was washed with 1 ml of the same mixture and centrifuged. The supernatants were combined, evaporated to dryness under reduced pressure, and weighed to determine the total content of lipid.¹⁹⁾

Partial Degradation—(1) Digestion with Pronase²⁰⁾: The sample was dissolved in 1/15 M phosphate buffer, pH 8.0, at a concentration of 2.5 mg/ml, and an aliquot (1.5 ml) of the solution was dialyzed overnight against the same buffer at 4°. To the dialyzed solution, 400 µg of Pronase was added and incubated for 1 hr at 35°. For assay of the hypoalbuminemic activity, mice received an intraperitoneal injection of 0.2 ml of the enzyme digest which had been dialyzed overnight against physiological saline.

(2) Digestion with TPCK-treated Trypsin: F-V was also digested with the TPCK-treated trypsin which had been prepared by the method of Ong *et al.*²¹⁾ To 6 mg of F-V dissolved in 5 ml of physiological saline, pH 8.0, 240 µg of the TPCK-treated trypsin was added and incubated for 16 hr at 4°. After addition of 240 µg of soybean trypsin inhibitor, the mixture was centrifuged for 2 min at 1000×*g* with Centriflo Membrane Cones (CF 25, Amicon). The inner solution was used for the bioassay.

(3) Digestion with DNase²²⁾: The sample was dissolved in physiological saline, pH 7.0, at a concentration of 2.5 mg/ml, and an aliquot (1.5 ml) of the solution was dialyzed overnight against the same physiological saline at 4°. To this solution, 3.3 mg of magnesium acetate and 20 µg of DNase were added and incubated for 1 hr at 35°.

(4) Digestion with RNase²³⁾: The sample was dissolved in physiological saline, pH 7.7, at a concentration of 2.5 mg/ml, and an aliquot (1.5 ml) of the solution was dialyzed overnight against the same physiological saline at 4°. To the dialyzed solution, 80 µg of RNase was added and incubated for 1 hr at 35°. Both preparations of DNase and RNase were confirmed to be free from the proteolytic activity toward casein at pH 7.5.

Extraction of Lipid—Lipid was extracted from F-V with butanol.¹⁰⁾ To 2 ml of the sample solution in physiological saline, containing F-V at a concentration of 2.5 mg/ml, 0.4 ml of butanol pre-cooled at –20° was added slowly and stirred for 1 min. The mixture was centrifuged for 20 min at 1000×*g* to remove the supernatant. The aqueous layer obtained was dialyzed overnight against physiological saline at 4°.

RESULTS

Determination of Albumin by Electrophoresis on Cellulose Acetate Paper

Fig. 1 shows that the serum albumin level is increased in proportion to the increasing concentration of the albumin added. Mouse albumin (0.05 ml), which was dissolved in physiological saline at concentrations of 10, 20, and 40 mg/ml, was added to an equal volume of the mouse serum

containing albumin at a concentration of 42.8 mg/ml. The albumin levels obtained were 26.6, 32.2, and 41.6 mg/ml, respectively. These results indicate that serum albumin levels of mice can be determined with reasonable accuracy by this method.

Purification

FIG. 1. Determination of Albumin Added to Mouse Serum by Electrophoresis on Cellulose Acetate Paper

Mouse albumin (0.05 ml), which was dissolved in physiological saline at concentrations of 10, 20, and 40 mg/ml, was added to an equal volume of the mouse serum containing albumin at a concentration of 42.8 mg/ml. An aliquot (8 μ l) of the mixture was applied to a strip of cellulose acetate paper for electrophoresis. Albumin levels were determined as described in "Materials and Methods". Each point represents the mean value of four or five determinations $\pm$ S.E.

TABLE I. Purification of Hypoalbuminemic Substance from Ehrlich Ascites Carcinoma Cells

	Yield (%)	ED ₂₀ (mg/kg)	Purification
F-I	100	1300	1
F-II	18.8	400	3
F-III	2.6	17	77
F-V	1.8	9	144
F-VII	0.3	3	433

ED₂₀ is the dose showing a decrease of 20% in serum albumin.

The supernatant (F-VI) obtained by ultracentrifugation at 110000 $\times$ g was subjected to gel filtration on Sephadex G-75 in the final step (Fig. 2). The main peak (fraction numbers 8 to 11, F-VII) showed a hypoalbuminemic activity. From 1.5 mg of F-VI, 0.95 mg of F-VII was obtained.

Table I summarized the purification of the hypoalbuminemic substance. The effective dose

FIG. 2. Gel Filtration of F-VI on Sephadex F-75

F-VI solution (1 ml, containing 1.5 mg of protein) in 0.9% NaCl containing 1% sodium cholate, was applied to the top of Sephadex G-75 column (1.1 $\times$ 33 cm) and eluted with 0.9% NaCl, pH 7.2, at a flow rate of 7 ml per hr. Fractions were collected in 1 ml per tube. The absorbance at 660 nm⁵⁾ was determined on sample diluted 20-fold.

F-VII

FIG. 3. Electrophoresis of F-VII on Polyacrylamide Gel Containing Sodium Dodecylsulfate

One hundred μ g of F-VII was applied to a tube. Electrophoresis was carried out in 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate with a constant current of 8 mA per tube at room temperature for 7 hr. Migration was from top (cathode) to bottom (anode).

(ED₂₀), which shows a decrease of 20% in serum albumin, was used instead of the specific activity for comparison of the activity and the purification of the preparation at each step. From 45 g of the packed cells, 15 mg of F-VII was obtained.

Purity and Molecular Weight of F-VII

Fig. 3 shows the pattern of electrophoresis of F-VII on polyacrylamide gel containing sodium dodecylsulfate. Only a single band was found on the gel stained with Amidoshwarz 10B. This band was also detected by staining with methylene blue. The molecular weight of F-VII was estimated to be roughly 53000 from a plot of the logarithm of the molecular weight *versus* the relative mobility to protein standards (Fig. 4).

FIG. 4. Estimation of Molecular Weight of F-VII by Electrophoresis on Polyacrylamide Gel Containing Sodium Dodecylsulfate

Electrophoresis was carried out as described in "Materials and Methods" and the legend to Fig. 3. (1) bovine plasma albumin (molecular weight 68000), (2) ovalbumin (43000), (3) trypsin (23000), and (4) cytochrome C (11700) were used as protein standards.

Chemical Composition

The results of amino acid analysis of F-VII are given in Table II. The numbers of amino acid residues were calculated on the basis of a molecular weight of 53000. Of the 17 kinds of amino acids detected, alanine and glycine were contained in fairly large amounts, whereas no cystine was found in the hydrolysate of F-VII. The contents of amino acid residues in F-VII were 14.6%. Identification of sugars in the hydrolysate of F-VII was carried out by thin layer chromatography. Authentic samples of galactosamine, glucosamine, galactose, glucose, and mannose had *Rf* values of 0.11, 0.21, 0.32, 0.37, and 0.42, respectively. The hydrolysate of F-VII gave three spots with *Rf* values of 0.10, 0.19, and 0.30, corresponding to those of galactosamine, glucosamine, and galactose, respectively. The hexosamine and hexose contents in F-VII were estimated to be 1.0 (as galactosamine) and 24.9% (as galactose), respectively. The numbers of residues of hexosamine and galactose are calculated to be 2 and 73, respectively, on the basis of a

TABLE II. *Amino Acid Composition of F-VII*

Amino acid	Content ($\mu\text{mol per mg protein}$)	(g residue per 100 g protein)	Residues per molecule ^{a)}	Nearest integer
Trp ^{b)}		1.89	5.4	5
Lys	0.123	1.58	6.5	7
His	0.031	0.42	1.6	2
Arg	0.031	0.48	1.6	2
Asp	0.103	1.18	5.4	5
Thr	0.070	0.70	3.7	4
Ser	0.114	0.99	6.0	6
Glu	0.131	1.69	6.9	7
Pro	0.019	0.18	1.0	1
Gly	0.178	1.02	9.4	9
Ala	0.171	1.21	9.0	9
Cys (half)	— ^{c)}	—	—	—
Val	0.085	0.84	4.4	4
Met	0.020	0.27	1.1	1
Ile	0.047	0.53	2.4	2
Leu	0.076	0.86	4.0	4
Tyr	0.021	0.34	1.1	1
Phe	0.028	0.42	1.5	2
Total		14.60		71

a) Based on molecular weight of 5300.

b) The content of tryptophan was determined on a separate sample by the ultraviolet spectrophotometric method.¹³⁾

c) Not detected.

TABLE III. *Biological Activities of F-V and F-VII*

Dose (mg/kg)	Serum albumin level (mg/ml)	Serum total protein level (mg/ml)	Leukocyte count ($\times 10^{-2}$)	Phagocytic index ($K \times 10^2$)
Control ^{a)}	44.1 $\pm$ 2.5	68.4 $\pm$ 1.8	94 $\pm$ 3	2.9 $\pm$ 0.2
F-V 12.5	29.8 $\pm$ 3.3 *	*56.8 $\pm$ 2.9 *	58 $\pm$ 6 *	4.9 $\pm$ 0.3 *
25.0	28.5 $\pm$ 3.9 *	55.7 $\pm$ 2.7 *	48 $\pm$ 8 *	6.6 $\pm$ 0.1 *
Control ^{a)}	48.1 $\pm$ 1.1	68.2 $\pm$ 1.2	91 $\pm$ 9	2.7 $\pm$ 0.4
F-VII 1.6	43.7 $\pm$ 2.6 **	67.8 $\pm$ 2.9	71 $\pm$ 3 **	4.4 $\pm$ 0.6 **
3.1	38.5 $\pm$ 2.0 *	63.3 $\pm$ 2.0 **	60 $\pm$ 7 **	5.3 $\pm$ 0.8 *

Assays were carried out 24 hr after intraperitoneal injection of F-V or F-VII.

Values are the means of four or five mice $\pm$ S.E.

a) Physiological saline, 10 ml/kg.

* Significant difference from control, $p < 0.01$.

** Significant difference from control, $p < 0.05$.

molecular weight of 53000. The nucleic acid contents in F-VII were estimated to be 24.6% for RNA and 28.9% for DNA. The lipid content in F-VII

was negligible. The sum of the contents of amino acid, carbohydrate, and nucleic acid is 94%.

Biological Activities

TABLE IV. *Decrease in Serum Albumin with Time after Administration of F-V*

Hours after injection	Serum albumin level (mg/ml)	
	Control ^{a)}	Treated
3	44.8±2.4	47.1±3.7
6	45.8±1.4	35.2±1.2*
12	47.3±1.8	32.2±2.8*
24	44.3±2.7	30.5±1.2*

Serum albumin levels were determined after intraperitoneal injection of F-V at a dose of 25 mg/kg. Values are the means of five mice ± S.E.

a) Physiological saline, 10 ml/kg.

* Significant difference from control, $p < 0.01$.

Table III shows the biological activities of F-V and F-VII determined 24 hr after administration. Significant decreases in serum albumin, serum total protein, and leukocyte count were observed with F-V and F-VII. The phagocytic activities of the reticuloendothelial system of mice were markedly stimulated by both fraction. These effects were also statistically significant against the control.

Table IV shows the time course of decrease in serum albumin after administration of F-V at a dose of 25 mg/kg. No decrease was observed at 3 hr, but at 6 hr the serum albumin levels decreased significantly ($p < 0.01$). More profound decreases were observed at 12 and 24 hr.

The effects of F-V and the unadsorbed fraction on the vascular permeability in mice are shown in Table V. When F-V was given to mice at a dose of 12.5 mg/kg, the leakage of dye was only 1 µg, whereas it was 31.7 µg at the same dose of the unadsorbed fraction. No hypoalbuminemic activity was observed with the unadsorbed fraction at this dose. This suggests that a vascular permeability-increasing substance may have concentrated into the fraction, being unadsorbed on DEAE-cellulose at pH 7.6. This fraction showed weak esterolytic and caseinolytic activities; its specific activities were 0.1 unit for tosyl-L-arginine methyl ester and 0.3 unit for casein (tyrosine used as reference standard). In contrast, F-V showed neither esterolytic nor caseinolytic activity.

TABLE V. *Effects of F-V and the Unadsorbed Fraction on Vascular Permeability*

	Dose (mg/kg)	Permeability (µg of Trypan Blue)
F-V	6.3	0.5±0.1
	12.5	1.0±0.5
Unadsorbed fraction	1.6	7.7±1.5
	3.1	16.5±2.5
	6.3	20.8±1.1
	12.5	31.7±2.7

The vascular permeability-increasing action was determined 20 min after injection into the skin of the abdomen of mice. Values are the means of three mice ± S.E.

Table VI shows that F-V displays both hypoalbuminemic and hypoproteinemic effects in the adrenalectomized mice as well as in the sham-operated ones, whereas it shows no leukocyte-lowering effect in the adrenalectomized mice. In order to confirm these findings, the following experiment was carried out (Table VII). Hydrocortisone was injected intraperitoneally into normal mice at a dose of 30 mg/kg once a day for six days, resulting in a marked decrease in leukocyte count. This effect was statistically significant ($p < 0.01$) against the control, whereas no significant change in serum albumin and serum total protein was observed.

Stabilities

F-V almost completely lost its hypoalbuminemic activity by heating in physiological saline for 1 hr at 60°. The effect of pH on F-V was studied in the following way. F-V was dissolved in physiological saline adjusted to pH 2, 4, 7, or 11 and dialyzed against the same physiological saline for 12 hr at 4°. After redialysis for 24 hr at 4° against physiological saline, pH 5.5, the pretreated F-V was assayed for hypoalbuminemic activity. The activity was decreased by 13 and 60% of the original at pH 11 and 2, respectively, whereas no loss of the activity was found at pH 4 or 7.

After the partial digestion of F-V with Pronase, the TPCK-treated trypsin, or DNase, its hypoalbuminemic activity almost completely disappeared, whereas no influence was observed after the

TABLE VI. *Effect of F-V on Adrenalectomized Mice*

	Serum albumin level (mg/ml)	Serum total protein level (mg/ml)	Leukocyte count ($\times 10^{-2}$)
Sham-operated			
Control	41.6 $\pm$ 3.7	72.3 $\pm$ 3.4	84 $\pm$ 6
F-V	32.0 $\pm$ 3.7 *	63.1 $\pm$ 2.6 *	37 $\pm$ 3 **
Adrenalectomized			
Control	43.5 $\pm$ 3.1	70.0 $\pm$ 2.5	82 $\pm$ 11
F-V	33.3 $\pm$ 3.6 *	61.2 $\pm$ 1.9 *	80 $\pm$ 14

F-V (12.5 mg/kg) was injected intraperitoneally into mice immediately after the operation and assayed 18 hr later. In control, physiological saline (10 ml/kg) was injected.

Values are the means of four mice $\pm$ S.E.

* Significant difference from control, $p < 0.05$.

** Significant difference from control, $p < 0.01$.

TABLE VII. *Effect of Hydrocortisone on Normal Mice*

	Serum albumin level (mg/ml)	Serum total protein level (mg/ml)	Leukocyte count ($\times 10^{-2}$)
Control	46.7 $\pm$ 1.9	70.8 $\pm$ 1.3	85 $\pm$ 3
Hydrocortisone	47.7 $\pm$ 1.2	75.1 $\pm$ 1.7	43 $\pm$ 3 *

Hydrocortisone (30 mg/kg) was injected intraperitoneally into mice once a day for six days. In control, physiological saline (10 ml/kg) was injected. Values are the means of five mice $\pm$ S.E.

* Significant difference from control, $p < 0.01$.

treatment with RNase or butanol.

DISCUSSION

A hypoalbuminemic fraction, possessing no vascular permeability-increasing action, was separated from mouse Ehrlich ascites carcinoma cells. The purified hypoalbuminemic substance, F-VII, was homogeneous in electrophoresis on polyacrylamide gel containing sodium dodecylsulfate. In a previous paper,¹⁾ a crude hypoalbuminemic substance, F-3, was obtained from homogenate of the cells at 0.66 saturation of ammonium sulfate of pH 4.7. This substance decreased serum albumin levels in mice over a period of 96 hr after administration at a dose of 50 mg/kg, and produced relatively profound hypoalbuminemia after 3 and 24 hr. F-VII obtained in this study decreased serum albumin levels 6 hr after its administration but not after 3 hr. These fin-

dings indicated that F-3 manifested the earlier (3 hr) and later (24 hr) effects separately and included at least two different principles. The hypoalbuminemic, hypoproteinemic, leukocyte-lowering and reticuloendothelial system-stimulating effects were all produced 24 hr after intraperitoneal injection of F-VII at a dose of 3.1 mg/kg. This suggests that F-VII may possess multiple physiological actions. It is known in general that when glucocorticoid is given repeatedly *in vivo* for a longer period, an involution of lymphoid tissue and a marked decrease in circulating lymphocytes are produced often in contrast to its administration for a shorter period. Recently, Kim and Kim,²⁴⁾ reported that in young adult rats receiving glucocorticoids in pharmacological amounts, the accumulation of proteins in liver was suppressed, and that in animals receiving multiple doses of glucocorticoid over a period of a few days, a more

pronounced inhibition in protein synthesis was observed. When F-V was given to the adrenalectomized mice immediately after the operation, its leukocyte-lowering effect disappeared. It may be possible, therefore, that the leukopenia is produced through the release of glucocorticoids from the adrenal cortex, and that F-V exerts its effect by stimulating the adrenal cortex to release glucocorticoids. These possibilities were further supported by the results that hydrocortisone produced the leukopenia when it was consecutively administered to the normal mice for six days. The results of the partial degradation suggest that the peptide and DNA moieties of the hypoalbuminemic substance may be participating in the hypoalbuminemic activity. The possibility that the lipid will directly participate in the biological activities of this substance may be excluded from the results of the present study.

There are several toxohormone preparations shown to possess different molecular weights. Matuo²⁵⁾ has recently re-examined the chemical properties of toxohormone and shown that this diversity in molecular weight was attributed to the degradation of the protein moiety by contaminating proteases during purification. The smallest species of toxohormone had a molecular weight of 10000 and was considered to be a basal construction unit, showing a liver catalase-lowering activity. Matuo has also shown that the distribution of toxohormone in cells was restricted to the nuclei, especially to chromatin. The toxohormone obtained from Rhodamine sarcoma was composed of 80% of protein and 20% of DNA, and it was suggested that the protein moiety might participate in the biological activity. So far we have not found any reports on the hypoalbuminemic activity of toxohormone.

Factors possessing immunosuppressive activity have been found in the serum of cancer patient,²⁶⁾ cell-free cancerous ascites fluid,²⁷⁾ and a material isolated from tumor cells.^{28a-c)} Motoki *et al.*^{28b)} have determined the chemical compositions of the factors obtained from Ehrlich ascites carcinoma

cells and reported that the ratio of protein, RNA, and DNA contents was 100:26:58 in the factor from chromatin^{28a)} and 100:1.2:0.0 in the factor from supernatant at 105000×*g*. The ratio in F-VII is 100:169:198 and appeared to be different from that in the immunosuppressive factors. It remains to be elucidated whether or not F-VII affects the host immune defense mechanism. A principle, which was partially purified from crude nuclear fraction of Ehrlich ascites carcinoma cells by the similar method for the purification of the hypoalbuminemic substance, decreased serum total protein and serum albumin levels in mice.* Therefore, the possibility that the hypoalbuminemic substance is a component derived from nuclear portion may exist. A crude hypoalbuminemic substance^{2a,b)} lowered the catalase activity of liver and reproduced a part of symptoms of cachexy by its administration to mice, however, further studies are necessary to clarify whether or not the hypoalbuminemic substance is a cachexy-producing one itself.

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