Journal of Chromatography, 275 (1983) 71—79
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1667

DETECTION OF THE CHANGES IN PROTEIN DISTRIBUTION IN RAT SERUM AFTER PARTIAL HEPATECTOMY USING TWO-DIMENSIONAL ELECTROPHORESIS UNDER NON-DENATURING CONDITIONS

TSUYOKI KADOFUKU*, TAKERU IIJIMA and TSUNEO SATO

Central Chemical Laboratory, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)

and

ICHIYO TEI and YOSHIAKI MAKINO

Department of Biochemistry, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)

(First received November 23rd, 1982; revised manuscript received February 2nd, 1983)

SUMMARY

The changes in rat serum protein distribution after partial hepatectomy were examined using two-dimensional electrophoresis, utilizing isoelectric focusing in polyacrylamide gel in the first dimension and pore gradient polyacrylamide gel electrophoresis in the second dimension. Drastic decreases in amount of protein were observed at more than twenty spot positions, and drastic increases in amount or newly appeared proteins were observed at eight spot positions. The amounts of albumin, immunoglobulin M and α_2-macroglobulin did not change relatively after hepatectomy.

The time course of the changes was examined using a densitometer, and it was observed that almost all the serum proteins changed drastically at 24 h after hepatectomy.

INTRODUCTION

The factors that trigger the proliferation of liver cells after partial hepatectomy in rat are not yet known with certainty. A series of specific substances are said to effect the proliferation of liver cells, but metabolic changes after partial hepatectomy possibly also act as a contributory causative factor in the stimulation of those substances. In order to define this question, the changes in the serum concentration of various metabolites after partial

0378-4347/83/$03.00

hepatectomy have been studied [1—4]. However, as for the changes in serum protein distribution after partial hepatectomy, little is known except for some proteins [2—5], due to poor resolution of the analytical techniques.

Manabe et al. [6, 7] described a two-dimensional electrophoretic technique which did not employ denaturing agents, and showed that human plasma proteins could be resolved into about 250 spots. Since this technique does not employ denaturing agents such as sodium dodecyl sulfate or urea throughout the electrophoretic run, it is suited to the analysis of mixtures of soluble proteins, maintaining their native physicochemical properties [8, 9] and their biological activities [10]. They also showed that this electrophoretic technique could be suitable for clinical purposes [11].

In the present report we show that the drastic changes in rat serum proteins, which may play an important role for the proliferation of liver cells, after hepatectomy can be analyzed by means of the two-dimensional electrophoretic technique.

MATERIALS AND METHODS

Reagents

Ampholines (pH 3.5—10 and pH 4—6.5) were obtained from LKB Produkter (Bromma, Sweden). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (all special grade for electrophoresis), Tris base, glycine and ammonium persulfate were from Wako Pure Industries (Tokyo, Japan). Coomassie Brilliant blue R-250 was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Hepatectomy and serum samples

Male rats (Wistar strain, 150 g) were used. Partial hepatectomy was performed under ether narcosis according to the method of Higgins and Anderson [12]. After hepatectomy the animals were maintained in our laboratory for 24—240 h. Food and water were provided ad libitum. At scheduled time intervals blood was taken from the descending aorta of each rat with a disposable syringe. The blood was left to stand at 4°C for 2 h, and then centrifuged at 3000 *g* for 10 min. Sucrose was added to the supernatant serum to give a concentration of 10% (w/v), and the serum samples were stored at −20°C until use.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was mainly carried out according to the method of Manabe et al. [6, 7]. First-dimension isoelectric focusing was performed on a gel column (14.5 cm × 0.5 cm I.D.). A 4% acrylamide (0.2% bisacrylamide) solution containing 2% Ampholine pH 3.5—10, 0.2% Ampholine pH 4—6.5 and 0.05% ammonium persulfate, was poured into the gel column. After gelling, the bottom ends of the tubes were covered with dialysis membrane held in place by O-rings, and the glass tubes were placed in a gel electrophoresis chamber. The electrode solutions were 0.04 *M* sodium hydroxide (cathode) and 0.01 *M* phosphoric acid (anode). An overlay solution (2% Ampholine pH 3.5—10, 0.2% Ampholine pH 4—6.5 and 5% sucrose, 50 μl)

was layered on top of the gel column and then serum samples (50 μl) were applied under the overlay solution. Electrophoresis was run at 2 mA constant current per gel column for 40 min and then at 460 V constant voltage for 20 h at 4°C. After electrophoresis the gel was pushed out by a rubber bulb and placed on top of the second-dimension slab gel without equilibration.

Second-dimension gradient polyacrylamide gel electrophoresis was performed with a slab gel apparatus which forms four slab gels 12 cm long, 16 cm wide and 0.4 cm thick. A 4—21% acrylamide linear gradient (0.2% bisacrylamide) containing a 0—10% sucrose gradient and a 0.05—0.025% ammonium persulfate gradient was poured in about 50 min at 4°C. Gelling occurred in about 2 h in a water-bath at 30°C. The gradient gel buffer was 0.14 *M* Tris—HCl (pH 8.9) and the electrode buffer was 0.05 *M* Tris—0.38 *M* glycine (pH 8.3). Electrophoresis was run at 50 mA constant current per slab gel for 22 h at 4°C. During the second-dimension electrophoresis, the electrode buffers in the anode chamber and the cathode chamber were exchanged continuously with each other at 40 ml per min using a peristaltic pump.

Measurement of the pH gradient

The first-dimension isoelectric focusing gel was duplicated for each sample. One of the gels was cut into 10-mm sections and these sections were placed in individual vials containing 2 ml of distilled water. These vials were capped and kept for 24 h at 4°C, then the pH was measured for each vial on a Horiba pH meter (Tokyo, Japan).

Staining and destaining

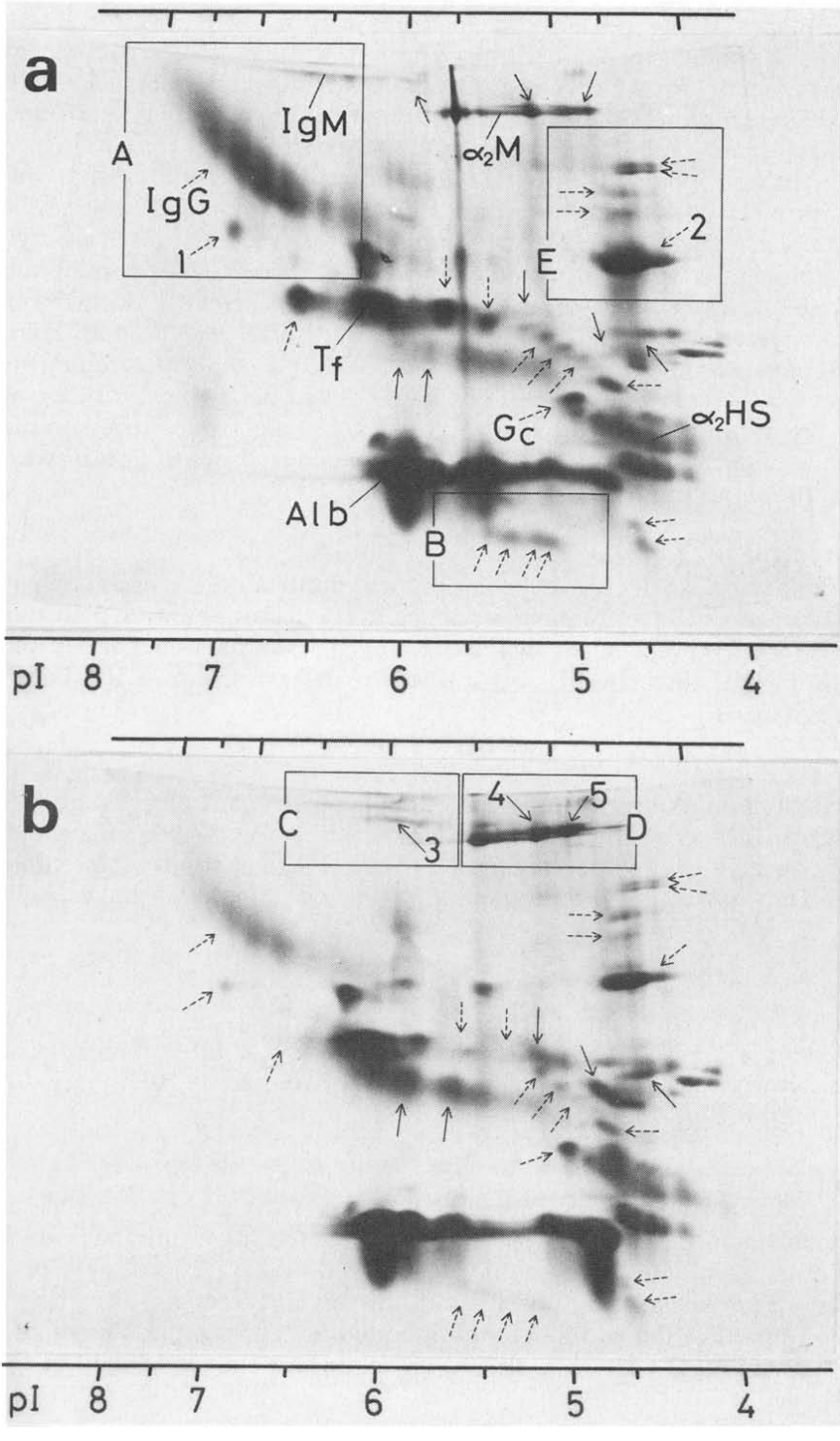
The gel was stained overnight in 0.025% Coomassie Brilliant blue R-250—7% (v/v) acetic acid—50% (v/v) methanol, and destained in 7% (v/v) acetic acid at 80°C for 4 h, then in two changes of 7% (v/v) acetic acid at room temperature for two days. During staining and destaining, the gel container was shaken gently.

Photography

Photography was carried out by placing the gel on top of a viewing box positioned under a 35-mm reflex camera. A Kenko Y-2 filter (Kenko Co., Tokyo, Japan) was attached to the camera and Fuji Neopan F 10D film (ASA 32, Fuji Photo Film Co., Tokyo, Japan) was used.

Densitometry

Densitometric quantitation of Coomassie blue-stained spots on the slab gel after two-dimensional electrophoresis was carried out using a Shimadzu dual-wavelength thin-layer chromatographic scanner CS-900 (Shimadzu Corp., Tokyo, Japan). Sample wavelength was set at 580 nm and reference wavelength was set at 750 nm. The densitometer was operated in "zig-zag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.


a
A
IgM
IgG
1
α2M
E
2
Tf
Gc
α2HS
Alb
B
pI 8 7 6 5 4
b
C
3
4
5
D
pI 8 7 6 5 4

RESULTS

Fig. 1a shows one of the two-dimensional electrophoretic patterns of normal rat serum proteins, before partial hepatectomy. The protein distributions of several rat serum samples were compared and the positions of the serum proteins were reproducible. Major serum proteins were located on the gel by comparing the patterns with those of human plasma proteins [13], and some of them are shown by letters in the figure. Fig. 1b shows a pattern of the two-dimensional distribution of rat serum proteins which was taken at 24 h after partial hepatectomy. Drastic changes of protein distributions in quantity were observed. The locations of the proteins whose positions or spot areas apparently changed after partial hepatectomy, are shown by arrows in the figure. The proteins that apparently decreased after hepatectomy were observed at more than twenty spot positions containing immunoglobulin (Ig) G, transferrin and G_c-globulin, and those are indicated by dotted arrows. The proteins that apparently increased or newly appeared after hepatectomy were observed at eight spot positions, and those are indicated by solid arrows. Some of the major serum proteins, which were tentatively identified as IgM, α_2-macroglobulin and albumin did not change relatively after hepatectomy.

The time course of the changes in the two-dimensional pattern of serum proteins was examined. Serum samples which were taken at 24, 48, 72, 96, 168, and 240 h after hepatectomy were subjected to the two-dimensional electrophoresis and the protein distributions were compared with those of normal serum pattern. The time-dependent changes at the gel sections of areas A, B, C and D (indicated in Fig. 1) are shown in Fig. 2. As shown by arrows in Fig. 2A and B, IgG, spot 1 and spots in area B were rapidly decreased at 24 h after partial hepatectomy, then gradually increased and almost recovered their original level at 96 h. In contrast, the proteins which drastically increased nor newly appeared after hepatectomy are shown in Fig. 2C and D. The protein of spot 3 (indicated in Fig. 1b) which did not exist in normal rat serum, appeared at 24 h after hepatectomy, gradually decreased, and then almost disappeared at 96 h (Fig. 2C). The proteins of spot 4 and spot 5 (indicated in Fig. 1b) also increased at 24 h after hepatectomy, and almost decreased to their original level at 96 h (Fig. 2D).

Fig. 3 shows the time-dependent changes at the gel sections of area E (indicated in Fig. 1a). The proteins in area E also changed after partial hepatectomy, but the changes were extremely slow. These proteins were minimal at 96 h and did not recover their original level even at 240 h after hepatectomy.

Fig. 1. Two-dimensional electrophoresis of rat serum proteins, (a) before partial hepatectomy, and (b) 24 h after partial hepatectomy. Arrows with a dotted line indicate the spots that apparently decreased after hepatectomy. Arrows with a solid line indicate the spots that apparently increased or newly appeared after hepatectomy. The positions of major serum proteins were located on the gel by comparing the patterns of rat serum proteins with those of human plasma proteins [13]. IgM = immunoglobulin M; α_1M = α_1-macroglobulin; IgG = immunoglobulin G; Tf = transferrin; G_c = G_c-globulin; α_1HS = α_1HS-glycoprotein; Alb = albumin.

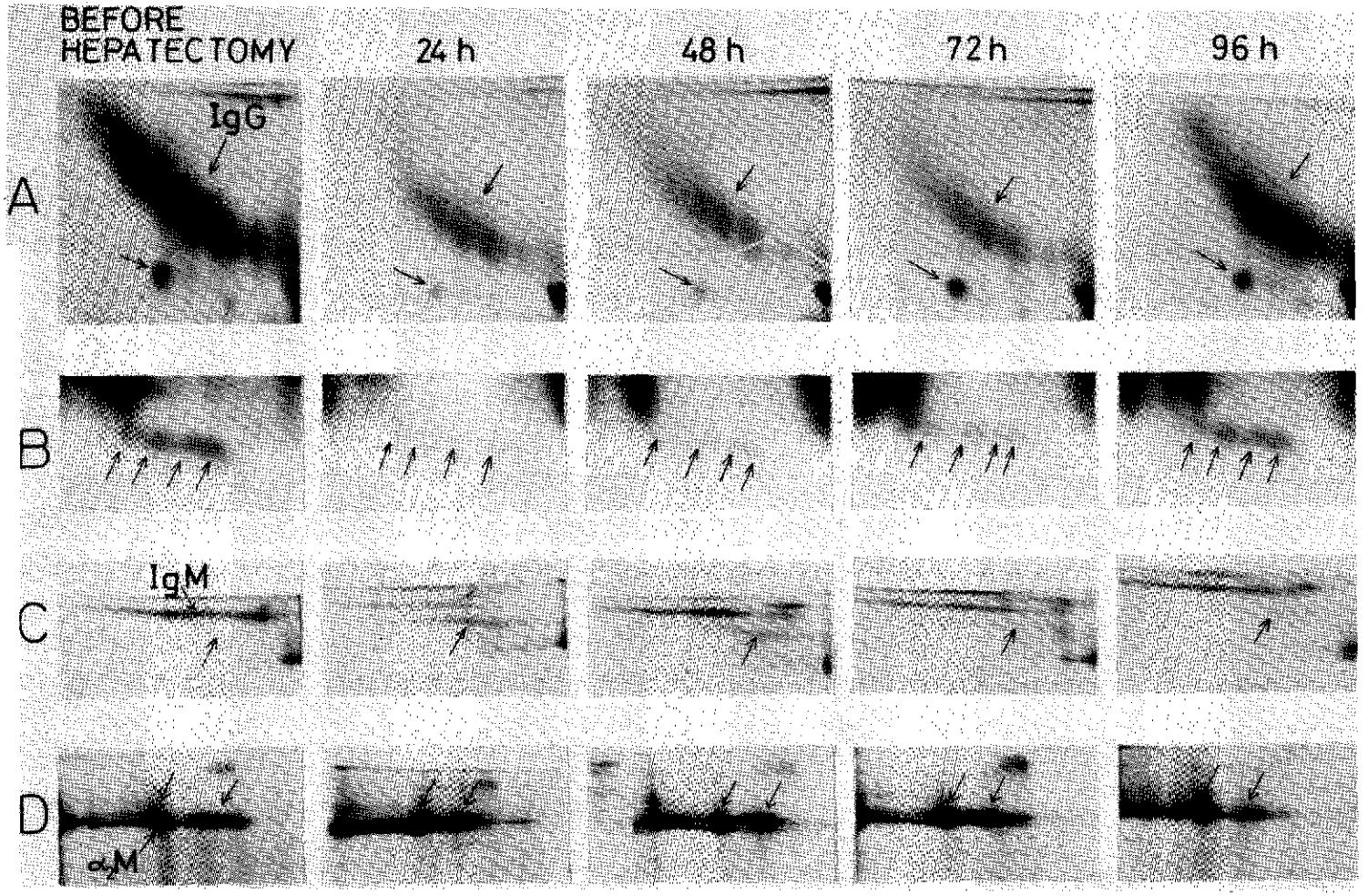


Fig. 2. Time course of the changes in the two-dimensional pattern of rat serum proteins after partial hepatectomy. Serum samples taken at 0, 24, 48, 72, and 96 h after partial hepatectomy were analyzed by two-dimensional electrophoresis. Time-dependent changes at the gel sections of areas A, B, C, and D (indicated in Fig. 1) are shown. Spot positions are indicated by arrows. IgG = immunoglobulin G, IgM = immunoglobulin M, α_2M = α_2-macroglobulin.

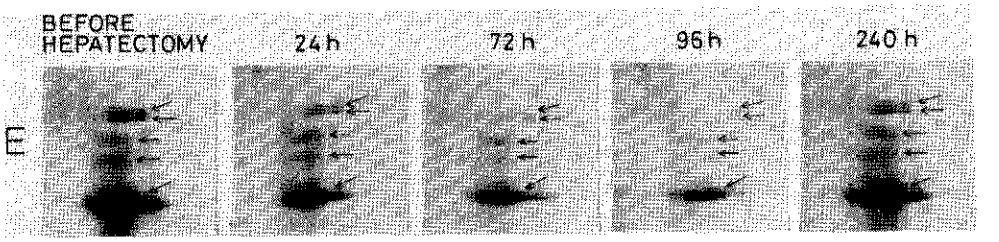


Fig. 3. Time course of the changes in the two-dimensional pattern of rat serum proteins after partial hepatectomy. Serum samples taken at 0, 24, 72, 96, and 240 h after partial hepatectomy were analyzed by two-dimensional electrophoresis. Time-dependent changes at gel sections of area E (indicated in Fig. 1a) are shown. Spot positions are indicated by arrows.

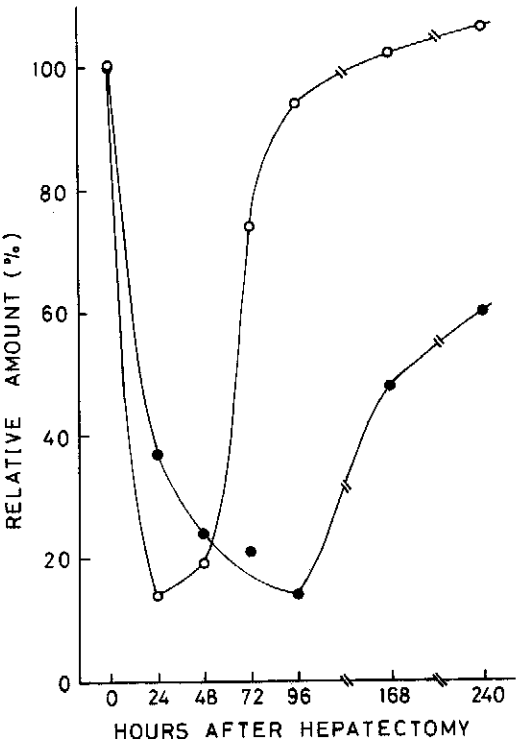


Fig. 4. Densitometric quantitation of spot 1 and spot 2 (indicated in Fig. 1a) after partial hepatectomy. Spots 1 and spots 2 on acrylamide slab gels were quantitated using a Shimadzu TLC scanner. (○—○), spot 1; (●—●), spot 2.

Densitometric quantitation of spots 1 and spots 2 on acrylamide slab gels was carried out with a Shimadzu TLC scanner. Fig. 4 shows the time courses of the quantity of spot 1 and of spot 2. A drastic fall in the amount of spot 1 at 24 h and of spot 2 at 96 h after hepatectomy was demonstrated.

DISCUSSION

Changes in serum proteins after partial hepatectomy have been studied by means of one-dimensional electrophoretic techniques, such as cellulose acetate membrane electrophoresis [2] and polyacrylamide gel electrophoresis [4]. However, these one-dimensional techniques could offer little information about the changes in the serum proteins which may be closely related to the proliferation of liver cells after partial hepatectomy. As shown in Figs. 1–3, the two-dimensional electrophoretic technique could detect the changes in serum protein distribution after hepatectomy. The time course of the decrease or increase of each protein spot also could be followed by means of the technique. The proteins which changed after hepatectomy can be divided into three types: type I includes proteins that drastically decreased in amount after hepatectomy (e.g. IgG, spot 1 and proteins in area B), type II includes those that newly appeared (e.g. spot 3) or drastically increased (e.g. spot 4 and spot 5), and type III includes those that did not change relatively (e.g. IgM, α_2-macroglobulin and albumin). We suggest that the proteins of type I and type II are closely related to the proliferation of liver cells after partial hepatectomy.

The electrophoretic technique employed isoelectric focusing in the first dimension and acrylamide pore gradient (4—21%) electrophoresis in the second dimension. The technique used no denaturing agent such as urea or sodium dodecyl sulfate throughout the run, thus equilibration of the first-dimension gel was not necessary. Further, when the isoelectric focusing gel was examined for the protein remaining after the second-dimension run, no Coomassie blue-stained band was observed. Therefore, comparison of spot area was possible since there was no loss of proteins during the course of the electrophoretic run. Densitometric quantitation of isolated spots (such as spot 1 and spot 2, see Fig. 1a) was readily performed using a TLC scanner. The quantitation will help to estimate the restoration of liver cells after partial hepatectomy.

Spot 3 was newly appeared protein after partial hepatectomy. It showed a relatively wide p*I* range (p*I* 5.5—6.5) and had an apparent molecular weight of about 900,000. The identification of this spot will not be easy since it can be either cellular protein or modified serum protein.

Spot 4 and spot 5 were rapidly increased proteins after partial hepatectomy. These proteins could also be found in AH-130 tumour-bearing abdominal ascites. Therefore, we suggest that these proteins play an important role in the proliferation of the cells. The molecular weights of these proteins were estimated to be about 800,000 and their isoelectric points were 4.9 (spot 5) and 5.1 (spot 4). Spot 4 and spot 5 were periodic acid—Schiff-positive proteins and this characterization may be useful for their purification.

REFERENCES

1 C.G.D. Morley and H.S. Kingdon, Biochim. Biophys. Acta, 308 (1973) 260.
2 W. Strecker, S. Silz, A. Salem and G. Ruhenstroth-Bauer, Horm. Metab. Res., 12 (1980) 604.
3 N. Nagasue, M. Kobayashi, A. Iwaki, H. Yukawa, R. Kanashima and K. Inokuchi, Cancer, 41 (1978) 435.

4 N.L. Coetzee, J. Short, K. Klein and P. Ove, Cancer Res., 42 (1982) 155.
5 R.E. Weesner, C.L. Mendenhall, D.D. Morgan, V. Kessler and C. Kromme, J. Lab. Clin. Med., 95 (1980) 725.
6 T. Manabe, K. Tachi, K. Kojima and T. Okuyama, Phys.-Chem. Biol., 22 (1978) 171.
7 T. Manabe, K. Tachi, K. Kojima and T. Okuyama, J. Biochem., 85 (1979) 649.
8 N. Takahashi, O. Oda, K. Kojima, T. Manabe and T. Okuyama, Phys.-Chem. Biol., 22 (1978) 279.
9 T. Manabe, N. Takahashi, K. Kojima, T. Shinoda and T. Okuyama, J. Biochem., 87 (1980) 451.
10 T. Kadofuku, T. Manabe and T. Okuyama, Phys.-Chem. Biol., 24 (1981) 319.
11 T. Manabe, T. Okuyama, A. Suzuki and A. Shigematsu, J. Chromatogr., 225 (1981) 65.
12 G.M. Higgins and R.M. Anderson, Arch. Pathol., 12 (1931) 186.
13 T. Manabe, K. Kojima, S. Jitsukawa, T. Hoshino and T. Okuyama, J. Biochem., 89 (1981) 841.