Acta Medica Okayama

Volume 20, *Issue* 5 1966 *Article* 3

OCTOBER 1966

Chemical analysis of soluble fractions from normal and autolysed rabbit liver by column chromatography

Saburo Yamamoto* Tadashi Aizawa† Satoshi Yoshikawa‡

Yasushi Matsuura** Yasuhisa Yamamoto††

*Okayama University,

†Okayama University,

‡Okayama University,

**Okayama University,

††Okayama University,

Chemical analysis of soluble fractions from normal and autolysed rabbit liver by column chromatography*

Saburo Yamamoto, Tadashi Aizawa, Satoshi Yoshikawa, Yasushi Matsuura, and Yasuhisa Yamamoto

Abstract

Chromatography on Sephadex G-200 was performed with the soluble fraction of homogenated rabbit liver, which was extracted with 0.1 M phosphate buffer containing 0.1 M NaCl. and the influences of autolysis on the soluble fraction of liver were also examined. The soluble fraction of liver was different from serum in molecular weight, in electrophoretic character and in components with sedimentation coefficients. The soluble fraction of liver was stable under the influence of Mg and K ions, and rather unstable in the presence of Na ions. Serum was fractionated in three main peaks. The soluble fraction of liver was fractionated in a similar pattern as of serum, but the first peak contained nucleic acid and lipoprotein. The second contained albumin. 32p radioactivity peaks of the stored sample appeared with change in patterns by autolysis from the original, and were observed wide based and continuous figures in retarded peaks. The correlations with the first peak and retarded peaks were represented by the analysis of phosphorus compounds and electrophoresis. In lipid analysis, both diglyceride and monoglyceride gradually decreased, and phospholipid pattern was observed to increase in retarded peaks by autolysis. Lipoprotein or lipid-albumin complex was gradually converted to smaller molecular weight compounds, and appeared in retarded peaks.

*PMID: 4227146 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 20, 203—214 (1966)

CHEMICAL ANALYSIS OF SOLUBLE FRACTIONS FROM NORMAL AND AUTOLYSED RABBIT LIVER BY COLUMN CHROMATOGRAPHY

Saburo YAMAMOTO, Tadashi AIZAWA, Satoshi YOSHIKAWA, Yasushi MATSUURA and Yasuhisa YAMAMOTO

Department of Surgery, Okayama University Medical School, Okayama, Japan (Director: Prof. S. Tanaka)

Received for publication, September 5, 1966

Many experimental methods for protein fractionation were presented in the past. Recently, the development of chromatographic techniques has made it possible to achieve extensive purified fractionation of proteins without recourse to complicated and extensive equipments. Gel filtration usually has been employed for this purpose, and is generaly used for the purification of enzymes[1,2], the separation of serum protein[3-5], or the fractionation of soluble vegetable proteins[6,7]. The molecular sieve principle forming the basis for gel filtration method has been used for the separation of substances with low molecular weight[8,9]. Molecules greater than approximately 200,000 in molecular weight do not penetrate the gel matrix and are eluted first. The smaller molecules appear in later effluents depending on the degree of their retention by penetration and subsequent displacement from the gel.

In this paper the investigations of the soluble fraction in rabbit liver on Sephadex G-200 column chromatography were carried out, and the influences of autolysis were examined by the storage of the sample for one or two weeks, and for comparison of the fractionated patterns of the soluble fraction in liver the rabbit serum was used.

EXPERIMENTALS

Rabbits of about 3.5 kg body weight were used after 60 hr intravenous administration of 10 mc ^{32}P sodium phosphate (purchased from Daiichi Pure Chemicals Co.). Serum was obtained by heart puncture under chloroform anesthesia, and liver was excised and washed out the blood with 0.15 M NaCl from portal vein. Rabbit liver was homogenized with 20 volumes of 0.1M phosphate buffer pH 7.2 containing 0.1 M NaCl. The homogenate was stored for 12 hr at 0～5°C and then ultracentrifuged at 50,000×g by the Type 40p Hitachi Preparative Ultracentrifuge. The supernatant was passed through minipore filter (HAWP

02500 25ea. HA 0.45 μ) to filtrate the smaller particles contained in the microsome fraction and then concentrated by ultrafiltration in Visking dialysing tube. This solution contained approximately 70 mg/ml protein, as judged by nitrogen estimation. This sample (original) was stored at 0~5°C, and was used to examine the influences of autolysis after one and two weeks later. Sephadex G-200 (purchased from Pharmacia Uppsale, Sweden) was washed 10 times with 0.01 M acetate buffer containing 0.01 M NaCl pH 6.2, and finer particles were removed by decanting after each wash. It was then allowed to swell for 3 or 4 days at room temperature. A column (1 × 190 cm) was packed with the Sephadex gel suspension by gravity flow. One ml volume of the sample was applied to the column and eluted at room temperature with 0.01 M acetate buffer containing 0.01 M NaCl pH 6.2. The flow rate was 6~8 ml/hr by gravity flow and the eluates were measured by the absorption at 280 mμ and 260 mμ in a Beckman DU spectrophotometer. Radioactivity of the eluates was determined by 2π gas flow counter fixing 0.2 ml of the eluates on metal planchets. The radioactivity in phosphorus compounds in the soluble fraction of liver was determined according to SCHNEIDER[10]. The recoveries of total ^{32}P activity after the chromatographic procedures were estimated 88.9 per cent in serum and 76.8 per cent in the soluble fraction of liver. Electrophoresis of each eluate was prepared with cellulose acetate (oxoid) as described by KOHN[11].

The eluates were separated into five fractions according to the elution curve of optical density at 280 mμ, radioactive patterns and electrophoretic features. Nitrogen contents of each of five fractions were determined by micro-Kjeldal method. Analytical ultracentrifugal analysis of chromatographed fractions was performed in the Type UCA 1 Hitachi analytical ultracentrifuge. Lipids were extracted by Folch's method from each fraction which were treated by lyophilization. Radioactivity of the extracted lipids (phospholipid) were determined by 2π gas flow counter. The lipid samples thus obtained were analyzed on thin layer chromatography Kiesel gel G plate. For developing solvent systems the followings were used; petroleum ether-ether-acetic acid 60 : 40 : 1 for neutral lipids[12], and chloroform-methanol-water 65 : 25 : 4 or chloroform-methanol-glacial acetic acid-water 65 : 25 : 8 : 4 for phospholipids[12,13]. Autoradiogram was formed on the thin layer plate for phospholipid. The spots on thin layer plate were detected by heating at 110°C for 10 min according to VOGEL[12] and SKIPSKI[13].

RESULTS

One ml each of rabbit serum and the soluble fraction of liver was analyzed as shown in Figs. 1 and 2. Serum protein appeared in three main peaks, and ^{32}P radioactivity was shown by dotted curve in Fig. 1. The soluble fraction of

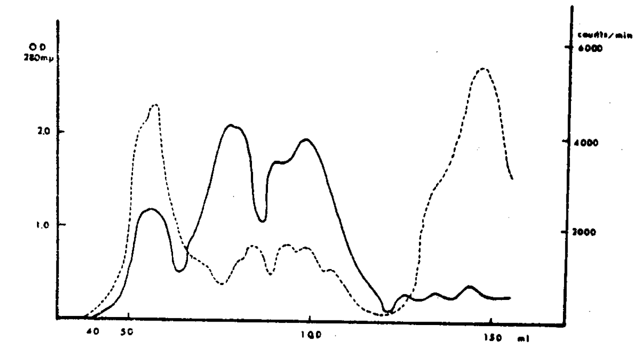


Fig. 1 Fractionation of serum protein on Sephadex G-200 column. One ml of rabbit serum was applied on 190×1 cm column, and eluted with 0.01 M acetate buffer pH 6.2, in the flow rate of 6—8 ml/hr at room temperature, dotted curve shows ^{32}P activity of 0.2 ml in each 2 ml of eluate.

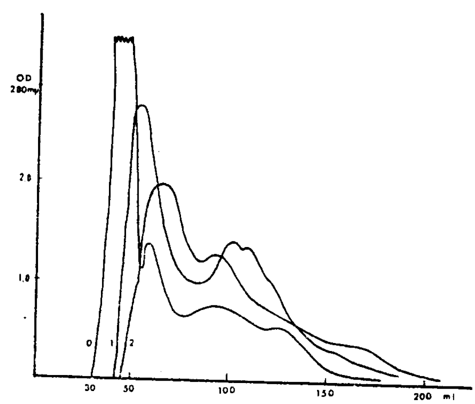


Fig. 2 Fractionation of soluble fraction in liver on Sephadex G-200 column. One ml of prepared sample was applied on the column (protein concentration was about 70 mg/ml). 0: original sample, 1: sample stored for one week, 2: sample stored for two weeks.

liver (original) was fractionated in two sharp peaks partially overlapped and another sloping curve from 60 ml. The initial elution point of serum protein was at 44 ml, and the soluble fraction of liver was at 30 ml. The influences of autolysis by storage of the original sample were observed in the elution curves 1 and 2 (Fig. 2). The initial elution point and the peaks were delayed from those of the original, respectively. The optical density of the original sample was measured at 260 mμ, and was higher than at 280 mμ in the elution from 30 ml to 50 ml, and from 50 ml to 130 ml the O. D. ratio of 260 : 280 mμ was stable about 0.7 : 1.0. The last elution from 130 ml showed higher optical density at 260 mμ than at 280 mμ. This is probably due to low molecular weight of RNA. The optical density of the stored sample was observed higher at 260 mμ than at 280 mμ throughout the elution. As shown in Tables I and II, nitrogen contents of the fractions IV and V in serum and the fraction V in the soluble fraction of liver were lower, while ^{32}P activity in these fractions appeared fairly high. Figure 3 illustrates ^{32}P activity curves of chro-

Acta Medica Okayama, Vol. 20 [1966], Iss. 5, Art. 3

Table 1 Nitrogen Content of Chromatographed Fractions

Fraction	Serum	Liver
I	1.32	3.94
II	1.88	2.62
III	3.90	3.98
IV	0.60	2.10
V	0.47	0.59

Nitrogen contents of fractionated serum and the soluble fraction of liver were determined by micro-Kjeldal method. Units were mg per each fraction. Fraction of serum : I ; 44–58 ml, II ; 59—68 ml, III ; 69—90 ml, IV ; 91—126 ml, V ; 127—166 ml (elution ml) Fractiion of the soluble fraction in liver (original) : 1 ; 30—42 ml, II ; 43—54 ml, III ; 55—74 ml, IV ; 75—118 ml, V ; 119—160 ml (elution ml)

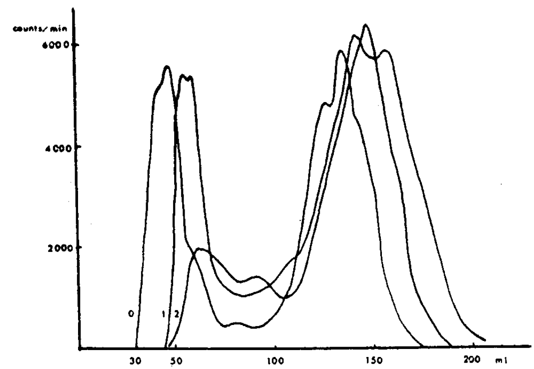


Fig. 3 ^{32}P activity curve of chromatographed soluble fraction of liver. Radioactivity was determined by using 0.2 ml eluate in each 2 ml eluate, and decay loss was adjusted. 0 : original 1 : sample stored for one week, 2 : sample stored for two weeks.

Table 2 ^{32}P Activity of Chromatographed Fractions

Fraction	Serum		Liver	
	counts/min × 10^3	%	counts/min × 10^3	%
I	357	30.9	351	12.3
II	118	10.6	549	19.3
III	148	12.6	216	8.5
IV	183	15.4	371	13.4
V	373	30.5	1364	46.5

Total radioactivity of each fraction was determined by 2π gas flow counter. Elution ml of each fraction is the same as in Table 1.

matographed soluble fraction in liver. The lipid ^{32}P activity of five fractions is shown in Table III. Radioactivity of phosphorus compounds in serum and the soluble fraction of liver was also determined as shown in Table IV. ^{32}P activity curves of the stored samples were observed with the delayed initial appearance and altered figures. Radioactivity of the first peak of the stored sample became gradually lower, and the retarded peaks were apparently higher than the original. As seen in Table III the ratio of lipid was about 30 per cent in the original sample and decreased by storage, and that of acid-soluble substrate was increased (Table IV), while the ratio of nucleic acid and phosphoprotein remained almost constant throughout the autolysis process.

In the electrophoresis diagram certain characteristic features were apparent (Fig. 4). The first peak of fractionated serum protein (Fig. 4, A) contained as

Table 3 ^{32}P Activity of Lipid in Chromatographed Fractions

Fraction	Serum protein		Soluble fraction of liver					
			original		one week		two weeks	
	counts/min	%	counts/min	%	counts/min	%	counts/min	%
I	1157	33.01	1276	65.00	1399	51.64	1252	45.34
II	1391	39.69	624	27.36	735	28.34	784	28.53
III	401	11.53	200	8.79	224	8.25	375	13.58
IV	193	5.50	152	6.67	196	7.55	213	7.72
V	361	10.23	28	1.23	100	3.85	134	4.87

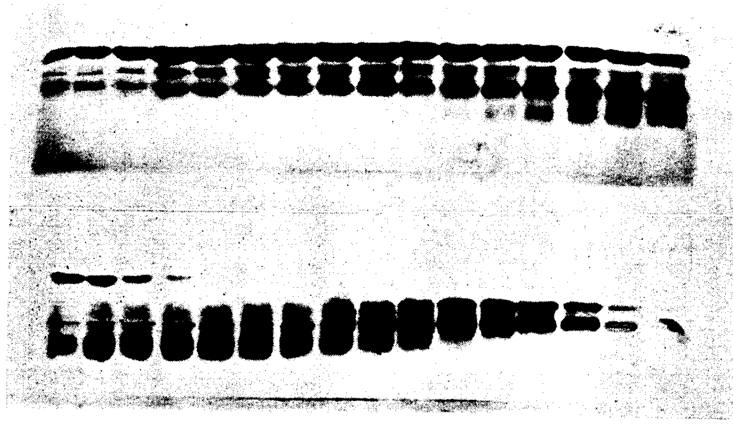
The lipid extracted from chromatographed fraction was dissolved with 0.5 ml of chloroform-methanol (2 : 1), radioactivity was determined by 0.1 ml of this solution and decay loss was adjusted. Elution ml of each fraction in serum and the original sample were the same as in Table I. Elution ml of one week stored sample: I; 40—56, II; 53—80, III; 81—104, IV; 105—140, V; 141—186. Elution ml of two weeks stored sample: I; 44—64, II; 65—82, III; 83—114, IV; 114—148, V; 149—204

Table 4 ^{32}P Activity of Phosphorus Compound in Serum Protein and the Soluble Fraction of Liver

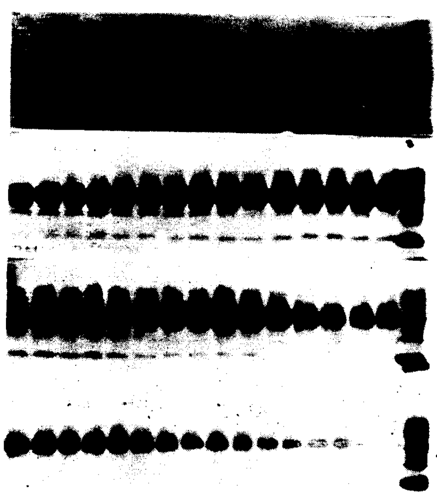
	Serum protein		Soluble fraction of liver					
			original		one week		two weeks	
	counts/min	%	counts/min	%	counts/min	%	counts/min	%
Acid soluble	1655	59.45	2165	59.00	1408	62.00	1120	64.81
Lipid	1089	39.22	1111	30.28	562	24.25	373	21.58
Nucleic acid	1	0.04	198	5.39	183	8.04	139	8.06
Phosphoprotein	33	1.16	195	5.31	121	5.70	96	6.66

One ml of rabbit serum and the soluble fraction of liver was treated as described by SCHNEIDER[10]. Decay loss was not adjusted.

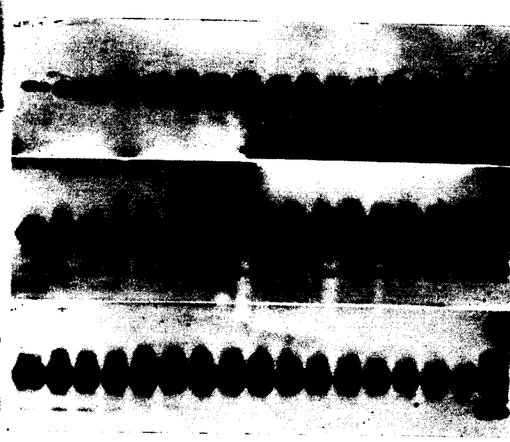
the major components α_1-globulin and lipoprotein. The second was dominated by γ-globulin and contained smaller amounts of β_1, α_2-globulins and albumin. The third contained mostly albumin. The last peak did not shift from the point of application, but radioactivity was shown with high peak in Fig. 1. The electrophoresis of soluble fraction of liver appeared in Fig. 4, B with altered pattern from serum and observed the influences of autolysis in Fig. 4, C. The first peak of the original sample remained at the application point but deeply stained by nigrosine. The band remaining on the application point of the original sample for 18 ml from the initial elution disappeared in the stored sample. This band was found with the different feature in the stored sample :



A



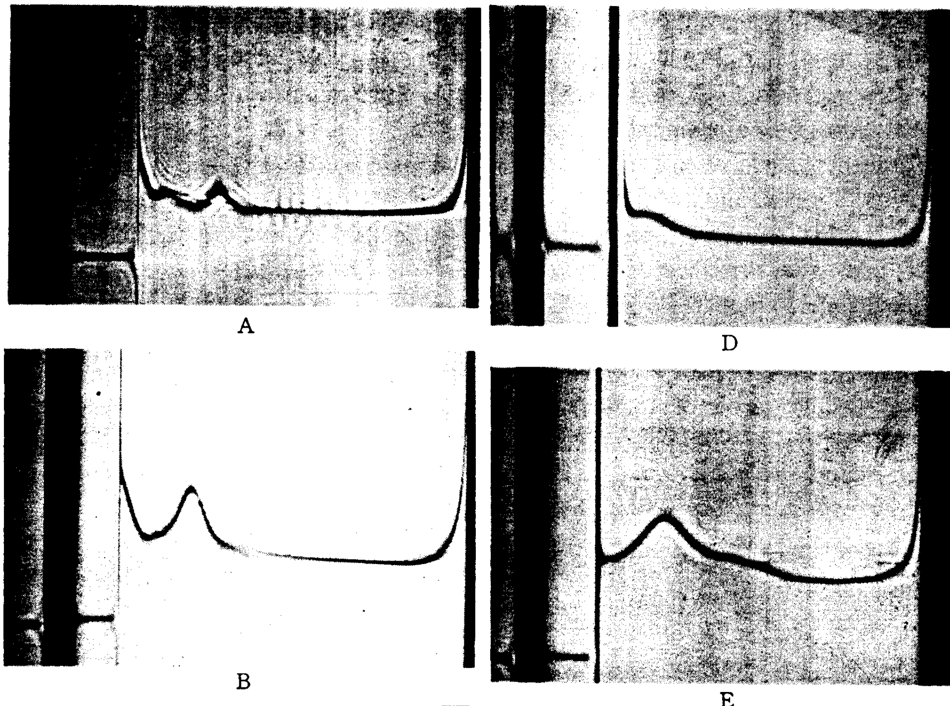
B



C

Fig. 4 Electrophoresis diagram of fractionated serum and the soluble fraction of liver. Electrophoresis was carried out by using cellulose acetate (oxoid) with 0.07 M veronal buffer pH 8.6, 0.6 mA/cm for 50 min and stained with 0.01% nigrosine. A: serum, B: original sample of the soluble fraction in liver, C: sample stored for two weeks of the soluble fraction in liver. Beginning of electrophoresis diagram is the initial elution of chromatography at 280 mμ, each application volume is 5 μl of fractionated 2 ml eluate, whole serum is applied for comparison to the right side on the figure of the soluble fraction in liver.

appeared in earlier eluate and removed through band a short distance from both side deeply stained and observed to increase gradually by storage. Albumin band appeared from 56 ml elution in original sample, and from 58 ml in the storage sample. α_2-Globulin band appeared from 56 ml elution in the original; 60 ml in one week, 62 ml in two weeks. α_1-Globulin band was found from 104 ml to 120 ml in the original, but disappeared by storage. γ-Globulin pattern



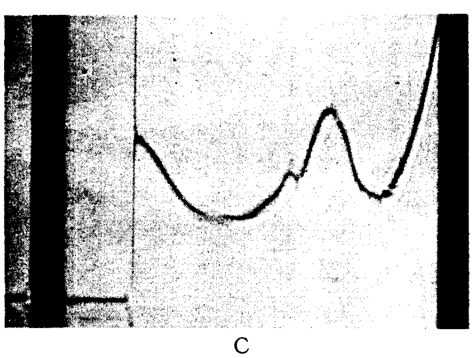


Fig. 5 Ultracentrifugal patterns of serum and the soluble fraction in liver. A: first peak of fractionated serum, 18-S and 14-S were observed at 51,200 rpm. B: second peak of fractionated serum, 9-S was observed at 60,000 rpm. C: third peak of fractionated serum, 6-S and 2-3-S were observed at 60,000 rpm. D: soluble fraction of liver, a low flat peak was observed at 20,000 rpm. E: soluble fraction of liver, two peaks were observed at 60,000 rpm.

was observed from 74 ml in the original; 82 ml in one week, 84 ml in two weeks. Both shifted through band to negative side over the band remaining on the application point. It was often divided into two or three bands within the extent of electrophoretic width of serum γ-globulin.

In analytical ultracentrifugal analysis of Fig. 5, the first peak of serum protein was found to contain two components with sedimentation coefficients of approximately 18-S and 14-S in the analytical ultracentrifuge of 51,200 rpm. The second contained one component with 9-S, and the third contained two components of 6-S and 2-3-S. The soluble fraction of liver was analyzed in the analytical ultracentrifuge without fractionation on Sephadex G-200 column, for the sample after chromatographic separation was so unstable and easily precipitated by addition of NaCl solution that concentration was impossible, thus observation of each peak being not represented. The peak was observed in the ultracentrifugal analysis of 20,000 rpm at first (Fig. 5, D), and two peaks appeared after 30 min of 60,000 rpm next (Fig. 5, E.)

Lipids in each fractionated peak of serum protein and the soluble fraction of liver were analyzed as shown in Fig. 6 on thin layer chromatography, and the fractions were the same with the fractions of lipid ^{32}P activity as described previously. The neutral lipids were contained in all the fractions but smaller amounts in the fraction V of original sample the same as in serum, and observed to the same rate distribution in each fraction of stored sample. The spot of Rf 0.4 (diglyceride) disappeared in stored sample. The spot of Rf 0.16 and 0.09

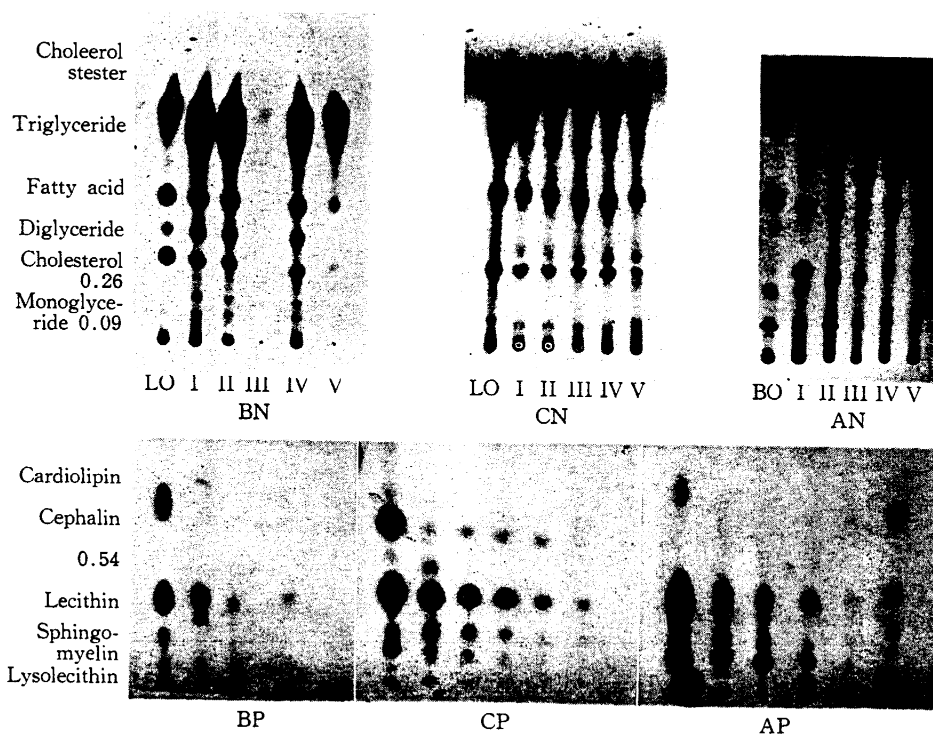


Fig. 6 Thin layer chromatography. N: neutral lipid, P: phospholipid, A: serum, B: original sample of the soluble fraction in liver, C two weeks stored sample of the soluble fraction in liver. Fractions I-V were the same as described previously, unidentified spots are indicated by Rf values, and phospholipid plate presented here is a radioautogram.
BO: sample extracted from whole serum, LO: sample extracted from the soluble fraction of liver which is the same material applied to Sephadex column.

decreased and disappeared by storage. The spot of Rf 0.28 in Fig. 6-CN was rather increased. The phospholipids were observed in the first peak of original sample, but in retarded peaks of the stored sample they were observed to increase gradually. The spot of Rf 0.34 in Fig. 6, CP appeared, even when unable to find in the original sample.

DISCUSSIONS

In the experiments reported here it was demonstrated that fractionation of the soluble fraction in liver could be accomplished by a simple chromatographic procedure using Sephadex G-200, and effects of autolysis on the extracts of liver were also studied. A preliminary test was made for the adequacy of the homogenizing solution by determining the protein concentration and lipid contents of the supernatant after ultracentrifugation. The 0.1 M phosphate buffer containing 0.1 M NaCl pH 7.2 was suitable and effective for the extraction of soluble fraction in liver.

FIREMAN *et al*[5]. reported about Sephadex G-200 chromatography that the first peak of fractionated serum protein was formed with the protein of molecular weight greater than 2000,000 and FLODIN[4] found the first peak of serum protein to be maintained mainly about 90 per cent with lipoprotein stained by Sudan black WHITAKER[14] studied the determination of molecular weight of protein by gel filtration method by using purified proteins and enzymes. He used 0.01 M acetate buffer pH 6.0 containing 0.1 M NaCl for the experiment. The buffer used in his gel filtration experiment was of a weak ionic strength of 0.01 M acetate buffer containing 0.01 M NaCl, for the strong ionic buffer made the effluent curve of the soluble fraction of liver elute without fractionation.

The column used here was the same length and diameter described by WHITAKER, so that the molecular weight of the eluted protein could be determined by the relationship between the elution volume and the molecular weight of protein, respectively.. AKERS[15] has described the equation formula about the relationship of elution volume and molecular weight on gel filtration method. These methods are dependent on the initial elution point, so that it is difficult to determine the molecular weight because structural proteins contain many kinds of protein.

In this paper the first peak of serum fractionation contained lipoprotein, and the first peak of the soluble fraction of liver contained greater molecular substance than in serum protein, respectively. The analyses of phosphorus compounds in serum and in the soluble fraction of liver revealed different results. Original sample of the soluble fraction of liver contained nucleic acid of about 5.4 per cent and lipoprotein containing phospholipid about 30 per cent in ratio. Autolysis elicited a decrease in lipoprotein and an increase in the acid-soluble substance but phosphoprotein and nucleic acid remained rather stable.

The radioactive patterns in serum protein was observed with high activity in the first peak and the last peak eluted about 130∼160 ml. This radioactivity of the first peak of serum protein paralleled mainly with the amount of lipoprotein containing phospholipid, and that of the last peak was probably dominated by the acid-soluble product. The high radioactivity of the first peak of the soluble fraction of liver was recognized depending on the contents of nucleic acid and lipoprotein, and the radioactive patterns in the retarded eluates were also considerable for the contained phosphorus compounds of protein bonded phospholipids as observed on the radioautogram of thin layer chromatography. But the fairly high ^{32}P activity of the last peak was probably due to the acid-soluble substrate, or low molecular weight RNA, or peptides, for the phospholipids in the fraction IV on radioautogram of original sample and lipid ^{32}P actvity in Table III were observed in smaller amounts. Nitrogen content was lower in fraction V of the soluble fraction of liver, about 0.59 mg. In this regard it is of interest to note that the influences of autolysis were mainly on the lipoprotein in the soluble fraction of liver.

The ultracentrifugal analysis of serum was successful as shown in Fig. 5-ABC. FLODIN[4] reported in the experiment of human serum fractionation by gel filtration that the first peak contained components with sedimentation coefficient of 19-S and 10-11-S, the second 7-S and 4-S and the third 4-S. In our experiment the rabbit serum contained 18-S and 14-S in the first peak, 9-S in the second and 6-S and 2-3-S in the third. The difference of species seems to account for this. The fractionated peak of the soluble fraction of liver was precipitated easily by concentration procedure. The fractionated peak of the soluble fraction of liver was precipitated by addition of NaCl solution, and the precipitate was dissolved by addition of Mg and K ions. This suggsts that the intracellular proteins were supported by Mg and K ions in a stable condition, and the influence of Mg ions was considerable mainly on nucleic acid bonded protein, and generally the intracellular protein is easily aggregated by fractionation procedure. The soluble fraction of liver was analyzed with ultracentrifugal analysis without fractionation by gel filtration as shown in Fig. 6-DE, and it was observed in smaller amounts of greater molecular weight substance that appeared low and flat peak on the figure of 20,000 rpm, and it was not possible to indicate the sedimentation coefficient. This would probably be a nucleic acid pattern. The peaks that appeared in the figure of 60,000 rpm were considered to be the compounds with the molecular weights near albumin, because the peaks were observed to shorten in height and base width with the time process, and to be separated to another flat peak shortly.

Electrophoresis by cellulose acetate (oxoid) readily yielded relatively sharp separated patterns with limited materials. A disadvantage of this method was the difficulty in the observation of lipoprotein that appeared with the wet and glanced band on the positive side of the application point. The electrophoresis diagram of serum protein was separated clearly (Fig. 5, A), and was observed with sharper figures than the paper electrophoresis as reported by FLODIN. Each fractionated sample of the soluble fraction of liver was separated on electrophoresis diagram, compared with that of serum. Most of the fractionated samples of the soluble fraction of liver revealed the band removing similarly with serum. γ-Globulin patterns of the fractionated original sample were observed with two or three bands within the extent of electrophoretic width of serum γ-globulin. α_2-Globulin shifted to the middle of α_2 and β_2-globulins of serum protein, and albumin to a shorter distance than that of serum. This would probably be due to the difference between serum protein and intracellular protein and the difference of the ionic strength. Autolysis changed the electrophoretic character of the soluble fraction of liver gradually. Albumin pattern of the soluble fraction in liver moved a greater distance in the electrophoresis of the stored sample. Generally, the effects of autolysis were to make albumin appear in early eluates and to be contained also in the retarded than the original. This was probably due to the autolysis converting the lipid-albumin complex to albumin and lipids.

Fractions III and IV of neutral lipids of serum protein contained smaller amounts of lipids than in fractions I and II. Phospholipids of serum were mostly contained in three main peaks, and smaller quantities in fraction IV. Neutral lipids of the original sample of the soluble fraction of liver appeared in five fractions with the same patterns, but fraction V contained a small quantity. Phospholipids of original sample mostly appeared in fractions I and II (first peak). Diglyceride decreased and disappeared gradually while the spot of Rf 0.16 and monoglyceride decreased. These lipid-bonded proteins were influenced by autolysis. Phospholipids appeared in retarded peak of stored sample. Alternatively there may have occurred autolysis of lipoprotein or lipid-albumin complex bonded diglyceride or monoglyceride or phospholipid to give smaller molecular protein or peptides or acid-soluble substrate.

These lipid patterns offered a suggestion that what kinds of lipids were bonding what sorts of protein or peptides.

SUMMARY

Chromatography on Sephadex G-200 was performed with the soluble fraction of homogenated rabbit liver, which was extracted with 0.1 M phosphate buffer containing 0.1 M NaCl. and the influences of autolysis on the soluble fraction of liver were also examined.

The soluble fraction of liver was different from serum in molecular weight, in electrophoretic character and in components with sedimentation coefficients. The soluble fraction of liver was stable under the influence of Mg and K ions, and rather unstable in the presence of Na ions. Serum was fractionated in three main peaks. The soluble fraction of liver was fractionated in a similar pattern as of serum, but the first peak contained nucleic acid and lipoprotein. The second contained albumin. ^{32}P radioactivity peaks of the stored sample appeared with change in patterns by autolysis from the original, and were observed wide based and continuous figures in retarded peaks. The correlations with the first peak and retarded peaks were represented by the analysis of phosphorus compounds and electrophoresis. In lipid analysis, both diglyceride and monoglyceride gradually decreased, and phospholipid pattern was observed to increase in retarded peaks by autolysis. Lipoprotein or lipid-albumin complex was gradually converted to smaller molecular weight compounds, and appeared in retarded peaks.

REFERENCES

1. MENDIOLA, L. and T. AKAZAWA: Partial purification and the enzyme nature of fraction I protein of rice leaves. *Biochemistry*. 3, 174, 1964
2. PAPAHADJOPOULOS, E., C. HOUGIE and D. J. HANAHAN: Purification and properties of bovine factor V; a change of molecular size during blood coagulation. *Biochemistry*. 3, 264, 1964
3. PRAGER, M. D. and I. C. ATKINS: Protein fractionation IV. Characterization of serum lipoprotein by chromatograpy on DEAE-cellulose. *Biochim. Biophys. Acta* 54, 405, 1961
4. FLODIN, P. and J. KILLANDER: Fractionation of human-serum protein by gel filtration. *Biochim. Biophys. Acta*. 63, 403, 1962
5. FIREMAN, P., W. E. VANNIER and H. C. GOODMAN: Immunochemical studies of human serum fractionated by gel filtration with Sephadex G-200. *Proc. Soc. Exp. Biol. Med.* 115, 845, 1964
6. HASEGAWA, K., T. KUSANO and H. MITUDA: Purification of the 11 S component of soyabean protein. *Agr. Biol. Chem.* 27, 878, 1963
7. MITUDA, H., T. KUSANO and K. HASEGAWA: *Agr. Biol. Chem.* 29, 7, 1965
8. PORATH, J. and P. FLODIN: Gel Filtration: A method for desalting and group separation. *Nature*. 183, 1657, 1959
9. FLODIN, P.: Dextran gels and their applications in gel filtration, Uppsale, 1962
10. SCHNEIDER, W. C.: Phosphorus compounds in animal tissues I. Extractions and estimation of deoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.* 161, 293, 1945
11. KOHN, J.: Small-scale membrane filter electrophoresis and immuno-electrophoresis. *Clin. Chim. Acta*. 3, 450, 1958
12. VOGEL, W. C., W. M. DOIZAKI and L. ZIEVE: Rapid thinlayer chromatographic separation of phospholipids and neutral lipids of serum. *J. Lipid Res.*, 3, 138, 1962
13. SKIPSKI, V. P., R. E. PETERSON J. SANDERS and M. BARCLAY: Thin-layer chromatography of phospholipids using silica gel without calcium sulfate binder. *J. Lipid Res.* 4, 227, 1963
14. WHITAKER, J. R.: Determination of molecular weight of proteins by gel filtration on Sephadex. *Anal. Chem.* 35, 1950, 1963
15. AKERS, G. K.: Molecular exclusion and restricted diffusion process in molecularsieve chromatography. *Biochemistry*, 3, 723, 1964