[Original]

The Interaction of Serum Proteins with Their Secreting Tissues, I. The Uptake of ^{125}I -labeled Rat Serum Albumin and γ -Globulin by Rat Liver and Spleen Tissue *in vitro*.*

By Shinzo Komatsu** and Yasuo Shibata***

Summary

1. Rat liver or spleen tissues were incubated *in vitro* with ^{125}I -labeled rat serum albumin or γ -globulin. Uptake of γ -globulin by the liver tissue was higher than that of albumin and the uptake by the spleen was converse.
2. It was observed that the uptake of exogenous proteins increased with time and the distribution of the incorporated proteins among subcellular fractions changed with time.
3. Microsomes prepared from rat tissue were incubated under cell-free conditions with ^{125}I -labeled rat serum albumin or γ -globulin. Liver microsomes bound larger amount of albumin than γ -globulin and in contrast, spleen microsomes bound larger amount of γ -globulin than albumin.

Introduction

Liver produces serum albumin at a considerable rate and reticuloendothelial tissues such as spleen produces γ -globulin. The mechanism for synthesis and secretion of these serum proteins are being studied by many authors. Constant levels of serum proteins in normal blood suggest some regulation mechanisms on their production or degradation, which have not been completely clarified.

Fahey demonstrated an increase in the degradation of γ -globulin after an administration of γ -globulin or in hyper- γ -globulinemia of mouse.¹⁾ Möller suggested a mechanism mediated by the specific antigen, i.e., the homologous specific antibody traps the corresponding antigen to eliminate its stimulating activity, resulting in a decreased synthesis of the antibody.²⁾ A feed-back regulation on albumin synthesis was claimed to be questionable in a perfusion experiment by Katz,³⁾ while an abundance of

albumin or dextran in liver interstitial space was reported to repress albumin synthesis.⁴⁾

In these *in vivo* experiments, any suppression on protein synthesis by the pre-formed same protein was not firmly observed, but recently some evidence to support a product-inhibition concept has been accumulated in *in vitro* experiments. Bank suggested that β chain synthesis was regulated by the amount of α chain in hemoglobin synthesis by rabbit reticulocyte.⁵⁾ Williamson reported a suppression of H chain synthesis by given immunoglobulin in mouse myeloma cells *in vitro*.⁶⁾

These *in vitro* studies lead us to suspect a feed-back regulation system on protein synthesis again. We have investigated an interaction of serum proteins (albumin and γ -globulin) with tissues responsible for their production (liver and spleen) *in vitro*, to detect any specific relationship between them. The transfer of ^{125}I -labeled rat serum albumin into liver and spleen tissues was compared with that of ^{125}I -labeled γ -glo-

* 血清蛋白とその分泌組織との相互作用, I. ^{125}I 標識ラット血清アルブミンおよび γ -グロブリンのラット肝臓, 脾臓組織への *in vitro* でのとりこみ.

** 小松信三, 昭和大学薬学部生理化学教室, 現: 鳥居薬品株式会社.

*** 柴田泰生, 昭和大学薬学部生理化学教室.

bulin upon incubation *in vitro*.

Materials and Methods

1) Purification and ^{125}I -iodination of albumin and γ -globulin.

Rat serum albumin was purified by CM-cellulose and γ -globulin by DEAE-cellulose column chromatography after ammonium sulfate fractionation from pooled sera. ^{125}I was reacted to these proteins in the form of ICl^{17} and a gel filtration through Sephadex G-25 followed to remove unreacted iodine. The specific radioactivity was 150,000 cpm/mg for albumin and 90,000 cpm/mg for γ -globulin and the both labeled proteins showed a single band in cellulose acetate electrophoresis.

2) Cell fractionation

Various subcellular fractions were prepared from normal rat liver and spleen. Homogenates (50 mM tris-buffer pH 7.4, 50mM KCl, 5 mM MgCl_2 , 0.25 M sucrose solution) were spun at 2,000 rpm for 10 minutes to sediment a debris fraction, spun at 10,000 rpm for 15 minutes to precipitate mitochondria, and spun at $105,000\times g$ for 1 hour to separate microsomes and the cell sap.

3) Incubation experiment of tissue sample with ^{125}I -protein

Normal male rat was sacrificed by bleeding under anesthesia, 3 aliquots of 200 mg of tissue sample were excised out from liver and spleen. The samples were minced by a knife, washed once with physiological saline and incubated at 37°C with 1.5 ml of basal medium (Chiba Kessei #199), to which was added approximately 5 mg of the labeled protein. Following the incubation, the tissue was separated from extracellular fluid by centrifugation after addition of 2 ml of cold saline and then washed once with 5 ml of saline. To investigate the distribution of radioactivity, the washed tissue was homogenized and the cell fractionation was done as described above. In addition, the debris fraction was stirred thoroughly in 4 ml of 0.25 M sucrose tris-buffer containing 0.5% DOC and spun at $105,000\times g$ for 1 hour to separate DOC-soluble supernatant fraction and DOC-insoluble debris.

Incubation of microsomes with labeled protein was carried out by a modification of the method for

cell-free incubation system⁸⁾ by adding 5 mg of labeled protein to 10 mg (protein) of microsomes. After the incubation, microsomes were collected by centrifugation at $105,000\times g$ for 1 hour and washed once.

Radioactivity was measured with an aliquot of each solution or with TCA-insoluble materials by a well type scintillation counter (Aloka TDC 5), the background of which was ca. 20 cpm.

Radioelectrophoresis was done in a disc tube (5×70 mm, 5% polyacrylamide gel, pH 8.0 tris-buffer) under 40 V/cm for 1.5 hour, followed by counting with a series of slab of gel 4 mm in width. The gel was stained also with Amino Black 10B.

Protein content of subcellular fraction was measured by Lowry's method.

Results

1) Uptake of ^{125}I -labeled protein by tissues

When liver or spleen tissue was incubated *in vitro* with ^{125}I rat serum albumin or γ -globulin at the given concentration of ca. 5 mg/200 mg of tissue wet weight, a considerable amount of radioactivity was transferred into the tissue, as illustrated in Table 1. These figures represent average values of the percentage of the total radioactivity transferred to the tissue in typical triplicate incubations. Statistically significant differences were observed between the two kinds of protein for each tissue. Liver took more γ -globulin than albumin and spleen conversely.

To remove contaminating extracellular radioactivity into the tissue fraction, washing was carried out with 5 ml of physiological saline. Radioactivities detected in the washings were 6.5% of the total given radioactivity for albumin and 6.2% for γ -globulin in the case of liver, and 3.6% for albumin and 3.5% for γ -globulin in the case of spleen. These figures would contain some radioactivity leaked from tissue fraction. Assuming that all the radioactivity found in the washing derived solely from media contaminants, a remnant of media in the tissue fraction after washing would be only less than 0.2~0.4%, being negligibly small compared with the figures in tissue, and thus the contamination by free ^{125}I -protein would have little importance.

Time course study on uptake of the labeled pro-

Table 1. Uptake of ¹²⁵I-labeled protein by rat liver and spleen tissue.

Tissue	Protein	Radioactivity (cpm × 10 ⁻³)		Relative Uptake (%)
		Given	Taken up	
Liver	Albumin	748.5 ± 2.7	51.0 ± 2.3	6.81 ± 0.26
	γ-globulin	383.4 ± 13.2	44.1 ± 1.2	11.49 ± 0.11
Spleen	Albumin	675.0 ± 10.7	56.1 ± 1.3	8.04 ± 0.10
	γ-globulin	432.0 ± 8.5	24.3 ± 0.1	5.62 ± 0.10

Each 3 samples of 200 mg wet weight tissue was minced and incubated with 1.5 ml of medium containing ca. 5 mg of ¹²⁵I-labeled protein for 1 hour. After incubation, the tissue was separated from the medium and washed once with saline. Radioactivity was measured with an aliquot of the solution sample and the whole precipitated sample. The given radioactivity is the sum of ¹²⁵I-radioactivities found in media, washing and tissue. Figures are the mean and the standard error of the mean from triplicate incubations.

Table 2. Increase of uptake of ¹²⁵I-labeled albumin by rat spleen tissue *in vitro*.

Incubation time (min)	Protein taken up (μg)	Relative uptake (%)
0	121.7 ± 11.8	2.7
20	211.8 ± 14.1	4.7
60	374.0 ± 11.8	8.3

Incubation was carried out under the conditions described in the legend to Table 1, except an incubation time. Radioactivities recovered in tissue were converted to the amount of protein by dividing its specific radioactivity. Figures are the mean and the standard error of the mean from triplicate incubations.

tein in the spleen-albumin system is presented in Table 2. During incubation the transfer of radioactivity into tissue appeared to increase gradually, indicating an active transfer or uptake of a given protein. Tissue without incubation (0 time) contained some radioactivity probably due to nonspecific absorp-

tion. Radioactivities detected in washings, 1.1 % at 0 and 1.5 % at 20 minutes, also showed an increase with time. These radioactivities would not be a remnant of media due to incomplete washing but rather an emission of once-incorporated substance from an interstitial matrix.

From the data in Table 1, calculations were made for the ratio of albumin/γ-globulin taken by each tissue, and 0.6 for the liver and 1.6 for the spleen were obtained. The evidence seems to demonstrate some selective uptake of the protein in each tissue, in other words, each tissue takes up relatively larger amount of the other kind of protein than the protein synthesized by itself.

2) Distribution of radioactivity in cell fractions

Table 3 shows a distribution of radioactivity among subcellular fractions after 1 hour incubation. Differences were observed between tissues and proteins. For example, γ-globulin mainly went into

Table 3. Distribution of ¹²⁵I radioactivity in cell fractions after 1 hour incubation.

Tissue	Liver		Spleen	
Protein	Albumin	γ-Globulin	Albumin	γ-Globulin
Sap	498 ± 27 (32.6)	1263 ± 66 (46.1)	341 ± 19 (18.3)	550 ± 36 (40.7)
Microsome	75 ± 8 (4.9)	112 ± 7 (4.1)	112 ± 13 (6.0)	60 ± 7 (4.4)
Mitochondria	130 ± 17 (8.5)	142 ± 8 (5.2)	248 ± 13 (13.3)	119 ± 10 (8.8)
Debris				
DOC soluble	610 ± 32 (39.9)	743 ± 4 (27.1)	950 ± 37 (50.9)	402 ± 26 (29.8)
DOC insoluble	216 ± 16 (14.1)	480 ± 7 (17.5)	213 ± 17 (11.4)	219 ± 20 (16.2)
Whole cell	1,530 (100)	2,740 (100)	1,865 (100)	1,350 (100)

Cell fractionation was carried out as described in "Materials and Methods" after the incubation. Figures, given in μg of protein/1 g of tissue, are the mean and the standard error of mean from triplicate incubations. Relative distribution is given in parenthesis.

Fig. 1. Gel filtration profile of postmitochondrial solution of tissue after 1 hour incubation with serum albumin.

Two ml each of postmitochondrial solution prepared by centrifugation for 15 minutes at 10,000 rpm of the homogenate of liver or spleen incubated with ¹²⁵I-labeled albumin was applied on Sepharose 6 B column (1.5 × 20 cm) in phosphate buffer (pH 7.0) following the addition of 0.5% DOC. The fraction size was 4 ml.

—, O.D.₂₆₀; ---, O.D.₂₈₀; ·····, ¹²⁵I radioactivity.

sap and DOC insoluble debris in liver, while albumin into mitochondria and DOC soluble debris fraction in spleen. A part of radioactivity detected in sap is considered to be a contamination of intercellular fluid. But even if sap fraction was omitted, the particle fractions were associated with a considerable amount of radioactivity and the tendency described above was true in both tissues.

In another experiment, the postmitochondrial supernatant from liver and spleen homogenate after the incubation with albumin was gel-filtrated through a Sepharose 6 B column (Fig. 1) and approximately 10% of radioactivity was recovered in a fraction of high molecular weight. Therefore, the radioactivities detected in the particle fractions in Table 3 are neither a remnant nor a contaminants from the sap.

The time course of the distribution of ¹²⁵I-labeled protein in tissue was studied in a spleen-albumin

system which showed a characteristic pattern of distribution as seen in Table 3. The results are given in Table 4 in terms of relative content which is a ratio of the amount of incorporated protein calculated from the radioactivity to the amount of pre-existed protein in each subcellular fractions determined by Lowry's method.

At 0 time, where the figures would indicate a distribution of radioactivity due to a passive absorption during the cell fractionation procedure, the particle fractions collected relatively much radioactivities. This tendency continued for the first 20 minutes and then, in the following 40 minutes an increase in sap and DOC-soluble debris fractions became apparent. These data would give some evidence supporting the uptake of protein by the cell itself through the interstitial space, excluding simple absorption of protein.

Table 4. Relative content and increasing rate of intracellular radioactivity in spleen cell after incubation with serum albumin.

Time (min)	Protein content (mg/g)	Relative content (%)			Increase (%)		Ratio of increase 60-0 min/20-0 min
		0 min	20 min	60 min	20-0 min	60-0 min	
Sap	49.1	0.22	0.34	0.70	0.12	0.48	4.0
Microsome	22.6	0.17	0.31	0.50	0.14	0.33	2.4
Mitochondria	12.9	0.71	1.49	1.92	0.78	1.21	1.6
Debris							
DOC soluble	55.0	0.51	0.84	1.72	0.33	1.21	3.7
DOC insoluble	21.6	0.45	0.72	0.99	0.27	0.54	2.0
Whole cell	161.3	0.38	0.65	1.15	0.27	0.77	2.85

Calculated on the basis that all radioactivity found in each subcellular fractions is associated with proteins. Figures represent percentage of the amount of radioactive proteins to that of the proteins determined by Lowry's method. The data are the means of triplicate incubations.

3) Characteristics of incorporated ^{125}I -protein in cell

The characteristics of ^{125}I incorporated into cell sap of spleen tissue upon incubation with the labeled albumin were analyzed at 60 minutes incubation. In gel filtration through Sephadex G-25 column, 70 % of radioactivity was associated with a large molecule fraction and 30 % with small molecules. The latter indicated a degradation of labeled albumin in the tissues. As illustrated in Figure 2, the disc electrophoresis profile of the large molecule fraction showed that most of the radioactivity moved with albumin, indicating a little exchange of ^{125}I among tissue proteins.

4) Binding of microsome with ^{125}I labeled serum protein

As shown in Fig. 1, a large molecule fraction of the spleen postmitochondrial supernatant in Sepharose 6B gel filtration was associated with unnegligible amount of ^{125}I (albumin) radioactivity. To confirm whether ribosomes of spleen bind other kinds of protein than albumin, a microsome preparation from rat liver or spleen was incubated with labeled albumin or γ -globulin under the cell-free condition described in "Materials and Methods." The microsomes showed a tendency to coprecipitate much more radioactivity than the tissue preparation, probably due to unspecific adsorption onto isolated particles. However, even in this condition the microsomes showed a selectivity. As illustrated in Table 5, microsomes from liver bound a larger amount of albumin than γ -globulin

Fig. 2. Radioactivity profile of disc electrophoresis of spleen sap after 1 hour incubation with ^{125}I -labeled albumin *in vitro*.

The sap was prepared from homogenate of spleen tissue after incubation. Disc electrophoresis was carried out as described in the text.

and those from spleen bound conversely. The A/G ratio defined above was high in liver and low in spleen. Microsomes bound the protein synthesized by themselves more than the other kind of protein. This property is just opposite to the data in Table 3, and rather reasonable considering their activities in protein synthesis.

Discussion

An uptake of serum proteins by tissues was discussed mainly as a process of their catabolism in several reports. In the entero-hepato cycle theory,⁹⁾ catabolism of serum proteins has been considered to

Table 5. Binding of ^{125}I -labeled protein with microsome.

Protein \ Microsome from	Liver (%)	Spleen (%)
Albumin (A)	7.1 ± 0.12	3.3 ± 0.02
γ -globulin (G)	6.8 ± 0.18	4.4 ± 0.35
A/G	1.04	0.75

Microsome preparation (10 mg protein) was incubated with 1.0 ml of media containing 5 mg of labeled protein at 37°C for 30 minutes. After incubation, particle was separated from media by $105,000\times g$, 1 hour centrifugation, and washed once with 1.5 ml of sucrose solution. Radioactivity recovered in microsome after subtracting that of blank incubation without microsome was converted to percentage to given radioactivity. Figures are the mean and the standard error of mean of triplicate incubations.

occur in a digestive tract and to result in a reutilization of amino acids after returning back into blood circulation. However, the evidence for the entrance of rat ^{131}I albumin into liver *in vivo*¹⁰⁾ and the degradation of serum proteins in some tissues was reported recently.¹¹⁾ It is likely that tissues directly reutilize amino acids derived from serum proteins by degradation as well as those from their constituent proteins.

How macromolecules such as serum proteins easily pass through a cell membrane had been a question, but it was solved by the "pinocytosis" theory. A story, phagosome which had eaten foreign substances changed its form to lysosome and the degradation of these substances followed, was argued in a rat liver-exogenous albumin system.¹²⁾ This hypothetical route would result in the reutilization of amino acids.

The present observation on the *in vitro* uptake of ^{125}I labeled rat serum proteins by rat tissues—the gradual increase of transfer of given radioactivity into tissue with a little ^{125}I exchange among tissue proteins and the translocation of radioactivity from the particle fractions to the other fractions in tissue during incubation—supports the concept of reutilization of serum proteins in tissue cells.

However, the tissue did not take any kind of serum proteins unselectively. Rat peritoneal mononuclear phagocytes were observed to take up only dena-

tured albumin but not native one.¹³⁾ Tissues or cells might have an ability of selection in uptake. In our experiment, the uptake of albumin by liver tissue was as low as 60 % of that of γ -globulin, while γ -globulin by spleen was converse. These facts show a selection mechanism, in other words, a tissue has a tendency to take up a smaller amount of its secreting protein than other kind of serum proteins. Although the uptake of albumin by liver tissue might not necessarily mean an incorporation into the albumin-producing cells, which was estimated to constitute 60 % of the liver cell population,¹⁴⁾ this selection is more interesting from the view point of regulation of protein synthesis than of the subject of their catabolism.

The protein entering the tissue where it is synthesized might have some effect on its own synthesis or secretion. There has been no evidence which shows a crosspoint between the secreting route of newly synthesized protein and the degrading pathway of taken protein, but a considerable amount of secreted immunoglobulin was found on the surface of its producing cells.¹⁵⁾

On the other hand, we found that, in contrast to these rejecting tendency of the tissues, microsome preparations of these tissues bound more own protein than the other kind of protein in cell-free incubation experiment. The finding that the microsomes bound more exogenous protein than in the case of tissue experiment was explained by a probable unspecific adsorption to naked microsomes, but in this circumstance the selection was evident. A microsome fraction should contain polysomes and secreting devices of the relevant protein, and recently mRNA from mouse myeloma cells was reported to bind myeloma protein specifically.¹⁶⁾ This fact allows us to consider a possibility of some effect of serum protein on protein synthesis in liver and spleen at the decoding level, in other words, this tendency is favorable to consider a regulation on protein synthesis by protein itself—a feed-back mechanism. We are now studying in cell-free system reconstituted from liver or spleen to find any suppressive effect of exogenous albumin or γ -globulin on cell-free protein synthesizing ability.

In contrast to this selection tendency of micro-

somes, tissue itself shows reversed selectivity in the uptake of serum proteins. It is not clear that the uptake of exogenous proteins by tissues directly means the uptake by their producing cells (it needs further histological studies including an autoradiographical examination to prove an entering of given protein into its synthesizing cells), but simply comparing the uptake of own secreting protein to that of another kind of protein, there seems to be some barrier in uptake. This barrier may be a reason that a feed-back regulation system has not been proved in *in vivo* experiments.

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要 約

健常動物での血清 alb, γ -glob 濃度の恒常性をそれら蛋白産生の feedback 調節によって解釈しようという試みは, *in vivo* 実験で証明できず, 高濃度時の異化促進などによって説明されている. しかし, 最近家兎網状赤血球でのヘモグロビン産生, マウス骨髓腫細胞での免疫 glob 産生について, cell-free 培養で完成蛋白(前者ではグロビン α 鎖, 後者では免疫グロブリン)が, 合成過程におけるアミノ酸配列順序のよみとりの段階を抑制または調節することが報告されている. このような *in vivo* と *in vitro* の結果の相違を考えると, まず組織による血清蛋白のとりこみ如何が問題となる. *in vivo* ではラットで肝臓などによる ^{131}I 標識 alb の異化が報告され, pinocytosis に始まる細胞内異化順路の推論も出されている.

われわれはラット血清 alb ならびに γ -glob を ^{125}I 標識して, ラット肝, 脾組織試料と *in vitro* 孵置し, これら蛋白の組織との相互作用をしらべた. 組織ミンス 200 mg 当たり 5 mg 程度の標識蛋白を *in vitro* 投与すると 37°C , 1 時間培養後, 1 回洗浄した組織部分にかなりの放射活性が移行する (alb の場合, 投与量の 6.8% (肝), 8.0% (脾), γ -glob の場合, 11.5% (肝), 5.6% (脾)). この移行は孵置時間とともに増大し, 細胞分画を行なって検討したところ, 放射活性の細胞内分布は臓器種, 蛋白種別に異なり, また, 時間別に変化した. さらに孵置後の細胞の上清分画の電気泳動分析で, 蛋白性 ^{125}I 放射活性の大部分が投与標識蛋白と等しい易動度を示した. 以上から *in vitro* での放射活性の組織への移行は吸着によるものでなく, 組織による標識蛋白のとりこみと推定し得る.

同時にその際, 両組織の蛋白種別とりこみの程度に差が見られた. Alb とりこみ量対 γ -glob とりこみ量の比を仮に A 対 G 比として表現すると, 肝臓組織ではこの値が 1 時間孵置で 0.6, 脾臓組織では 1.6 となり, 両組織ともそれぞれの産生蛋白種を他より少なくとりこむ結果が得られた. 一方, 両組織よりミクロゾームを調製して, cell-free 条件で標識蛋白との孵置を行なうと, 30 分孵置で A 対 G 比は肝臓の場合 1.04, 脾臓の場合 0.75 と, それぞれ自己の産生蛋白種を多く結合する傾向が示された. これはマウス骨髓腫蛋白が骨髓腫細胞の mRNA と特異的に結合するとの他家の報告と並行する結果であり, 細胞下レベルでは自己産生蛋白種との親和性が強い.

以上から, 肝臓および脾臓の組織に蛋白種弁別, とりこみ抑制機構の存在が考えられ, これがまた他家による *in vivo* 実験での feedback 調節の証明不成功につながったと推定する.

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