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Studies on the metabolism of connective tissue

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Studies on the metabolism of connective tissue*

Masaaki Takatori

Abstract

A granuloma pouch was formed on the back of rats by the original method of SELYE. Seven days when granuloma tissue reached its maximum, 35S labeled ChS, 59Fe labeled ChS-Fe, labeled ferric ammoninum citrate and colloidal 198Au were injected into the pouch and their absorption and organ distribution examined and compared with the results in the case where 59Fe labeled ferric ammoninum citrate and colloidal 198Au were injected into the gluteal muscle. 1. When 35S labeled ChS was injected into the granuloma pouch, radioactivity of the organs per gram tissue was high in the kidney, liver, bone marrow and spleen, in descending order. The maximum activity was seen 12 to 24 hours after injection, which is slow compared to the results obtained by KISHIDA in intraperitoneal and oral administration. 2. The absorption of Ch S-Fe by pouch where the iron is enveloped by the large ChS molecule, is slower than that of ferric ammonium citrate, an inorganic compound. 3. The uptake of Fe from the blood by bone marrow is larger when the increase of blood Fe ion concentration is slow, rater than when the increase is rapid. 4. When conoidal 198Au is injected into the pouch and injected into the" gluteal muscle, the 198Au is phargocytozed by the reticuloendothelial system organs, the liver showing the largest uptake among all organs. 5. In the intramuscular injection of colloidal 198Au and 59Fe labeled ferric ammonium citrate, radioactivity of pouch fluid is lower than that of blood. However, the difference between the two is less in the case of colloidal 198Au. 6. In the granuloma ponch, radioactivity of the abdominal wall proves to be greater than that of the dorsal wall.

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STUDIES ON THF METABOLISM OF CONNECTIVE TISSUE

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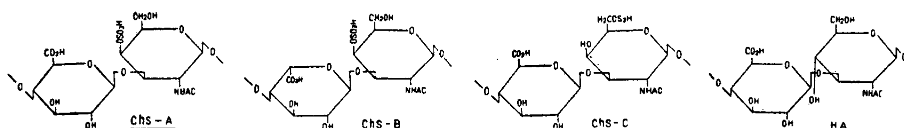
Together with the great accumulation of knowledge on the biochemistry of the cell in the last ten years, studies on the intercellular substances have made also a great progress. Following Morgani's idea of pathologic anatomy and Virchow's histopathology, KLEMPERER and others forwarded the idea of collagen diseases based upon the histopathology of intercellular substances and drew the attention of the medical world to connective tissue. Connective tissue can morphologically be divided into the cellular component, fibrous component, and amorphous colloidal interestitial component (ground substance), but functionally it is considered as one unit. Located between the blood vessels and parenchyma cells, its most important function is to make an environment most suitable for cellular life, such as by maintaining a constant cellular environment, transfer of nutritional substances and metabolic products. The importance of connective tissue hitherto only considered in its subrole— as a structural support of organ tissue— is thus being recognized. However, quite some time will be needed before the definite mechanism can be explained.

The ground substance of connective tissue histologically shows a strong metachromasia to basic dye and colloidal iron, and is also stained a homogeneous red by the periodic acid Schiff's reaction (PAS reaction). Metachromasia is shown by acid mucopolysaccharide, but the PAS positive substance is not acid mucopolysaccharide[1] but a complex carbohydrate-protein complex[2]. The stainability of the ground substance is decided by these two carbohydrates, but from their quantity they cannot be the main components of the ground substance. HAKOMORI reported the carbohydrate content of ground substance to be from a few per cent to 20 per cent at most, the main component other than water and salts being a protein called "non-collagen protein". He further states that this protein, acid mucopolysaccharide and neutral complex carbohydrate tightly combine to form a gigantic ultramicroscopic network throughout the body.

From resent cell culture observations it is clear that these acid polysaccharides and procollegen are synthesized by fibrodlasts, and it may be assumed that the non-collagen protein and neutral complex carbohydrates are also made by the fibroblasts. Further, the mast cell is assumed to be a semicellular endocrine

organ closely related to homeostatic activity. The acid mucopolysaccharide synthesized by the fibroblast is said to play the main functional role of ground substance in regards to the protein which is the main component of the ground substance. At present it is known that hyaluronic acid (hereafter abbreviated as HA), chondroitin sulfates (hereafter abbreviated as ChS) A, B and C, heparin, etc, are acid mucopolysaccharides. Further, their sugar composition, the struc-

Appendix I. Structure of chondroitin sulfate. (repeating unit)



Appendix 2. Comparison of the properties of chondroitin sulfate A, B and C.

	Chondroitin sulfates		
	A	B	C
[a] D	28 to − 32°	55 to − 63°	16 to − 22°
Ethanol fraction Precipitating Ca salt	30 to 40%	18 to 25%	40 to 50%
Hydrolysis by testicular euzyme	+	−	+
Anticoagulant activity	−	+	−
Electrophoretic mobility	∼ − 13	∼ − 13	∼ − 13
Hexosamine	chonbrosamine	chondrosamine	chondrosamine
Uronic acid CO_2	equimolar to hexosamine	equimolar to hexosamine	equimolar to hexosamine
Uronic acid carbazole	equimolar to hexosamine	half mol. /mol. hexosamine	equimolar to hexosamine
Reducing sugar equivalent after 1 h at 100° with NH_2SO_4	15 %	32 %	22 %
Sulfate after 1 h at 100° with NHCl	50 %	50 %	50 %

ture, chemical and physical characteristics of the main components are known. Especially, there are many clinical studies[26] in the case of ChS, with the three isomers being isolated, and the structure and characteristics thoroughy investigated[4-25].

In this experiment, ^{35}S labeled ChS was injected into the granuloma pouch of a rat and the absorption from the pouch and distribution in the organs was

studied. Also the absorption and organ distribution of ^{59}Fe labeled ChS-Fe, ^{59}Fe labeled ferric ammonium citrate, and ^{198}Au labeled gold colloid when injected respectively into the granuloma pouch and gluteal muscle was compared.

EXPERIMENTAL ANIMALS AND GRANULOMA POUCH FORMATION

Male rats weighing from 110 to 130 g were used. The animals were given water *ad libitum* and a constant amount of Oriental food pellets.

The granuloma pouch was formed according to the original method of SELYE[27]. That is, after shaving and sterilizing the dorsal skin of a 120 g rat, 25 ml of air was injected into the subcutaneous tissue in the area between left and right scapula to form an air pouch. Into this pouch 1 ml of one per cent croton oil was injected. KISHIMOTO[28] and SHIGEMASA[29] have reported on the histology and absorption rate of the granuloma pouch thus formed. According to these reports since granulation is maximum on about the seventh day after injection of croton oil, in this experiment the seeventh day granuloma pouch was used.

OBSERVATIONS

ABSORPTION AND ORGAN DISTRIBUTION OF ^{35}S LABELED ChS AFTER BEING INJECTED INTO THE RAT GRANULOMA POUCH

1. *Experimental Materials*

The method of BOSTRÖM[30] was used for the extraction of ^{35}S labeled ChS.

Table I. Purification of ^{35}S Labeled Chondroitin Sulfate (by Boström)

Rat 40 animals (each injected $Na_2\,{}^{35}SO_4$: 8.0×10^7 c. p. m.)
↓ — after 24 hours decapitated and exsanguinated.
Rib cartilage collected
↓ — dried with pure alcohol
Powder (3.2 g)
—
Boiled (15 min). Chilled (5 C)
↓ — 10% NaOH added
Shaken (16 hr)
↓
Centrifugated
↓
Concentrated
↓
Centrifugated at high speed (12000 rpm)
↓ — alcohol added (3 volumes)
Precipitated
↓ — Acetic acid added
Precipitated
↓ — sodium carbonate added
Supernatant dialized (in cold room 3 days)
↓
Deproteinated
↓ — NaCl half saturated ethyl alcohol (4 volumes)
Chondroitin sulfate sodium salt (151.2 mg)

First a carrier was added and then 1.2 mg of ^{35}S labeled Na_2SO_4 was injected intraperitoneally into 80 rats of approximately 120 g body weight (8.0×10^7 c. p. m. by infinite thinnes method). Next, 24 hours after the injection when the turnover of ^{35}S to ChS reached the maximum in the rat rib cartilage, the animals were sacrificed by exanguination and ChS extracted from the rib cartilage by Standberg's modification of the JORPES method[31]. The amount of extracted ^{35}S labeled ChS in the form of Na salt was 302.4 mg.

Of this material, 4 mg were added to boiling $4N$ HCl and hydrolyzed for 4 hours, precipitated in the form of benzidine sulfate by the benzidine method (ROSENHEIM DRUMMOND method[32]) and the radioactivity measured. The specific activity was 5.5×10^3 c. p. m.

2. *Methods*

Twenty four male rats, 110—130 g body weight, each bearing the 7 day granuloma pouch, were divided into 6 groups of 4 rats each and 0.5 ml of a 2 per cent solution of ChS labeled with 10 mg of ^{35}S (5.5×10^4 c. p. m.) was injected into the granuloma pouch of each animal. The respective groups were sacrificed 3, 6, 12, 24, 48 and 72 hours later by exsanguination, the amount of blood measured, and the organs weighed. Organ tissues were treated by the wet oxidation method[33] to oxidize organic sulfur compounds to the inorganic sulfur salt. The ^{35}S containing material was precipitated by benzidine sulfate, placed in stainless steel sample plates and after dehydration the radioactivity was measured. Radioactivity was measured on a Kobe Kogyo 2π Gasflow Counter, Model PR-123, the plateau[34] being 1250 volts. The data were corrected for autoabsorption, weight and recording day.

3. *Analysis of Experimental Methods*

a) *Extraction of ^{35}S labeled ChS* — For the extraction of ChS there are the neutral salt method and the alkali solution method. Both methods have their good and bad points, but as there was a danger of contamination in this case where radioactive S was used, the comparitively-simple Jorpes method[31] was used. According to JORPES, 16 hours extraction in a cold room using 2 per cent NaOH will give good results with no separation of the sulfate or decomposition on ChS. However, even by the Jorpes method a certain amount of separation of the sulfate cannot be avoided. A 2 per cent kaolin solution was used for deproteinization (Table I).

The extracted ChS (sodium salt) gave the following analytic figures: N: 2.97 % (KJELDAHL's method), S: 5.85 % (EGAMI's method[35]), [α] -26.80.

b) *Granuloma pouch formation* — As the status of the granuloma pouch is the basis of this experiment, in order to maintain constant conditions a large number of rats were used and a selection was made. The ganuloma pouch was

formed according to the Selye's original method as stated in the previous section. In the walnut sized pouch, granuloma tissue is formed on the inner wall which is covered with subcutaneous connective tissue. According to KISHIMOTO[28] the granuloma pouch is at its maximum growth on the seventh day after injection of croton oil. Therefore, the 7 day granuloma pouch was used in this experiment.

c) *Preparation of ^{35}S containing material* – As the energy of beta rays from ^{35}S are very weak (0.167 MeV.) a large part of the radiation of ^{35}S in the material is absorbed. However, this can be measured by changing the S into inorganic sulfate and precipitating this as the benzidine sulfate of $BaSO_4$, or by reducing it to H_2S, which by being changed into methylene blue by the St. Lorant's method[34], can be absorbed on active charcoal[37]. In the present experiment the inorganic sulfate was measured, the wet oxidation method[33] with nitric acid and hydrogen peroxide being used. (There is also the wet oxidation method using Pirie's reagent[38], in which decomposition is rapid and complete, but as this method is somewhat dangerous, the author used the HNO_3-H_2O_2 method.) The sample is placed into a test tube, 2 ml of concentrated HNO_3 are added, allowed to stand overnight, then this is heated to 100°C over a water bath to allow complete decomposition, after cooling 1 ml of 30 per cent H_2O_2 is added, and the mixture is heated to 100°C over a water bath. Usually this is sufficient for decomposition, but when decomposition is insufficient, the addition of H_2O_2 and heating is repeated. The author repeated this procedure four to five times. When sufficiently decomposed, the mixture is evaporated to dryness, after cooling 2 ml of HCl (1 : 4) are added, again evaporated to dryness, extracted with 2 ml of warm HCl (1 : 4) and filtered. To the filtrate 10 ml of benzidine reagent (0.1 *M* benzidine dihydrochloride in 0.4 *N* HCl) are added with stirring. After standing at room temperature for 20 minutes, aceton is added to the final concentration of 25 per cent, left in an icebox overnight to allow precipitation, filtered and absorbed on a WH 107 73G radiation glass filter, a homogeneous precipitate layer is made directly on filter paper, placed in a stainless steel plancet and gradually dried with an infrared lamp, and the radioactivity measured. The amount of precipitate used for measurement is calculated by the titration method[39], and the specific activity obtained by correcting the values for autoabsorption according to the chart previously prepared on the instrument used throughout this experiment (Fig. 1).

The theoretical formular[34,37,40] for the curve in Fig. 1 can be expressed as $\frac{I}{I_0}=\frac{1-e^{-ad}}{ad}$, where $\frac{I}{I_0}$ is the ratio between the obtained value (I) and the corrected value (I_0), a : is the absorption coefficient and d : the surface density. Further correction for the amount of sample, obtained by quantitative analysis, gave the

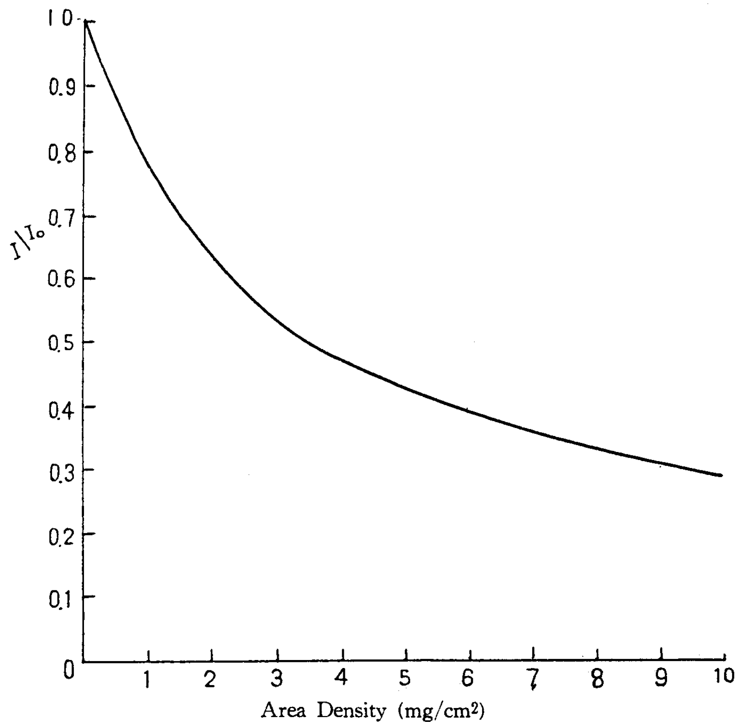


Fig. 1. Correction curve for autoabsorption of benzidine ^{35}S sulfate.

true radioactivity of the sample. When measuring the radioactivity of a sample, various other corrections stated by ZUMWALT between the samples is the important factor in this experiment, various corrections stated by ZUMWALT were not performed here as the measuring conditions in this experiment were constant.

4. *Results*

Radioactivity of samples measured is usually expressed as specific activity. This means the amount of radioactivity contained in a unit amount (usually 1 mg) and for convience expressed in counts per minute. In the present experiment this is expressed as the count of radioactivity per gram of organ tissue. Further in order to keep the relative percentage of error under 3 per cent, measurements were carried up to over 500 counts and the count per minute calculated from the total time.

In Fig. 2, the curve shows c. p. m. per gram organ tissue. In other words, the total wet weight of the organ obtained at the time of autopsy is multiplied by the counts per gram tissue. However, at the later value may be complicated by such factors as exsanguination, and errors at the time of autopsy the count

per gram weight is mainly used.

a) At three hours after injection, radioactivity is seen in all organs. Bone marrow shows the highest total organ activity, followed by muscle, but by gram organ tissue the spleen, liver and kidney show the highest counts. The abdominal wall of the granuloma pouch shows a higher count than the dorsal wall. In regards to granuloma pouch fluid, 57.3 per cent of the injected amount (5.5×10^4 c. p. m.) was recovered at three hours, showing that 42.3 per cent had been absorbed through the pouch wall.

b) After six hours, the organ showing the highest radioactivity per gram tissue was the spleen, followed by the liver and kidney. Although the radioactivity of each organ is about twice the amount seen at three hours, the general tendency is the same.

c) After 12 hours, the kidney shows the highest radioactivity. The radioactivity of the liver is increased to twice that of the spleen and the activity of the bone marrow is greatly increased. The radioactivity of the pouch fluid is decreased to 1/5 of the initially injected amount.

d) After 24 hours, the radioactivity of the kindney, liver, blood, brain and muscle showed a decrease compared to the 12 hours value, with the pouch walls and pouch fluid also showing a decrease. However, in the other organs the radioactivity reached the maximum values, especially the bone marrow showed a high radioactivity. The radioactivity of the pouch fluid showed a logarithmic decrease and the pouch wall a linear decrease.

e) After 48 hours, except for the kidney and bone marrow all organs showed a marked decrease in radioactivity, the respective values being the same or lower than those of the third hour after injection. The kidney and bone marrow also showed a marked decrease, but the degree was less than that seen in the other organs.

f) After 72 hours, the radioactivity of the organs was further decreased. The radioactivity of the pouch fluid was 4.7 per cent of the injected amount.

5. *Summary*

a) Throughout the whole period of observation, the kidney, liver, blood, heart, brain and muscle showed their highest radioactivity 12 hours after injection. Radioactivity was highest in the kidney.

b) The spleen, lung, stomach and intestine, bone marrow and skin showed their highest radioactivity 24 hours after injection. Radioactivity of the bone marrow showed the highest radioactivity.

c) The radioactivity of the blood showed a marked increase from the sixth to twelfth hour after injection, with the thereafter decrease being rapid. However, the maximum level was lower than that of the kidney, liver or spleen.

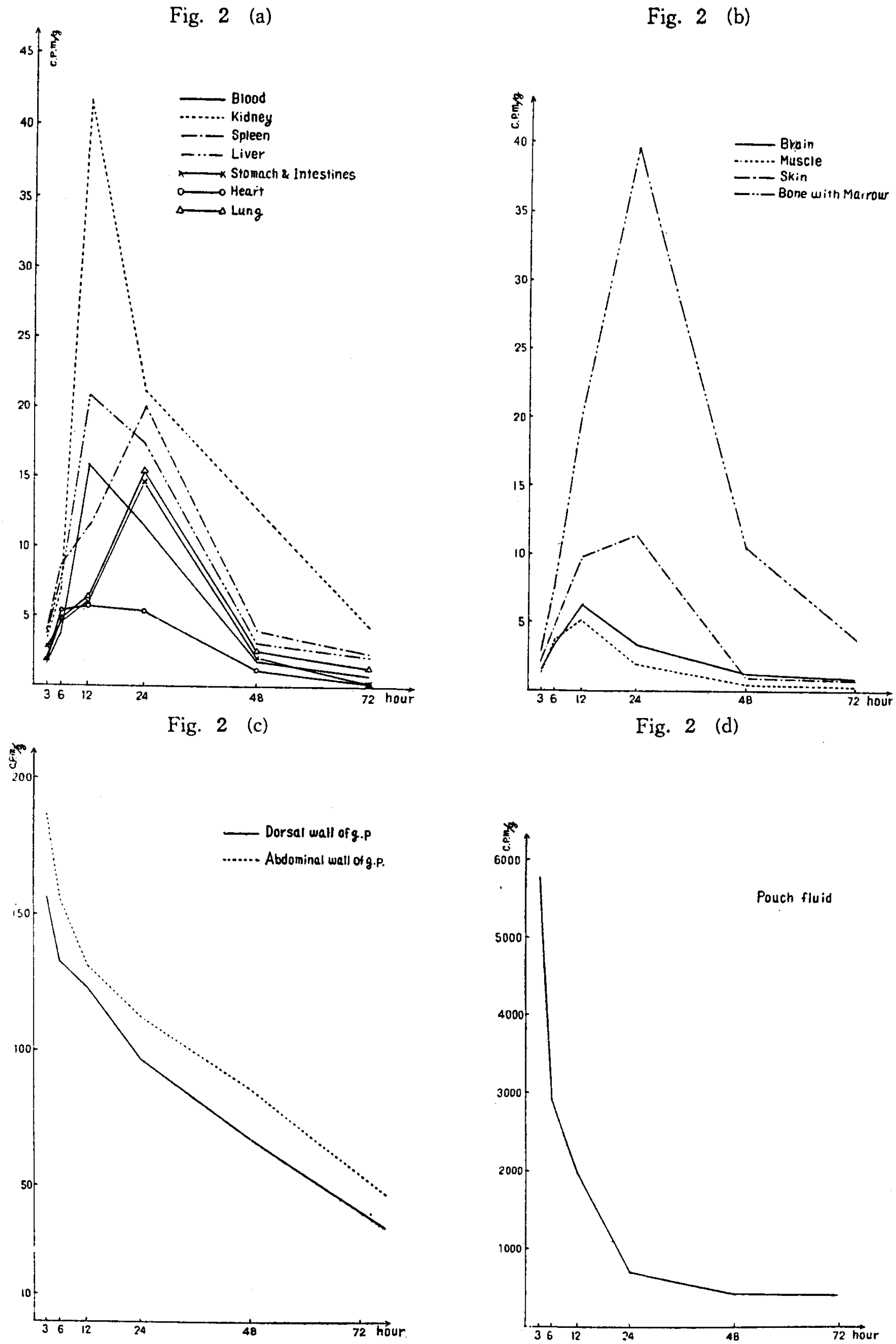


Fig. 2. Absorption and distribution of ^{35}S labeled chondroitin sulfate from the rat granuloma pouch.

d) Except for the pouch wall and pouch fluid, the kidney and bone marrow were the only organs showing a large amout of radioactivity 48 hours after injection.

e) The abdominal wall of the pouch showed a higher radioaetivity per gram tissue than the dorsal wall.

f) The radioactivity of the pouch fluid revealed a logarithmic decrease.

THE ABSORPTION AND ORGAN DISTRIBUTION OF ^{59}Fe LABELED ChS INJECTED INTO THE RAT GRANULOMA POUCH

1. *Materials*

The ^{59}Fe labeled ChS-Fe was prepared by the laboratory of the Dai Nippon Seiyaku Kabushiki Kaisha. The pH was 6.8—8.5, and the Berlin blue reaction was negative, showing it to have no free Fe ions. The assumed structure is;

$$[(C_{13}H_{19}O_{14}NS)Fe(OH)_3]_n \qquad n=130-160$$

Most of the Fe was assumed to be protected by ChS, being in the form of a colloidal Fe $(OH)_3$ surrounded by ChS. Further, besides the colloidal bonds, common bonding between the sulfate of the ChS and Fe^{++}, and a condensation between the hydroxy of the ChS and Fe^{+++} was assumed.

2. *Methods*

The Fe labeled ChS-Fe was injected into the 7 day granuloma pouch of 24 rats, prepared according to the original methed of Selye. The amount of ChS-^{59}Fe injected into each animal (corrected for day of injection) was;

^{59}Fe: $3\,\mu c/1.5$ ml $=1.64\times10^6$ c. p. m. (gamma rays)
Fe: 6 mg/1.5 ml
ChS: 30 mg/1.5 ml
pH = 8.5

In accordance with the method stated in the previous section, groups of four animals were sacrificed at different times after injection, 1 g of tissue from each organ homogenized in a glass homogenizer, and the gamma rays measured on a Kobe Kogyo Well type scintillation counter-PS-lB. The half life of ^{59}Fe is 45.1 days, with 0.271 and 0.462 MeV. beta rays and 1.289 and 1.098 gamma rays. In this experiment, as gamma rays were measured, the only corrections made were for those of day of experiment, 'autoabsorption in this case being negligible.

3. *Results*

The results of measurements are shown in Fig. 3. In examining the radioactivity per gram tissue of the organs it has been found that; a) the activity after three hours, is high in the kidney, blood, bone, spleen and liver, in decreasing

Fig. 3. (a)

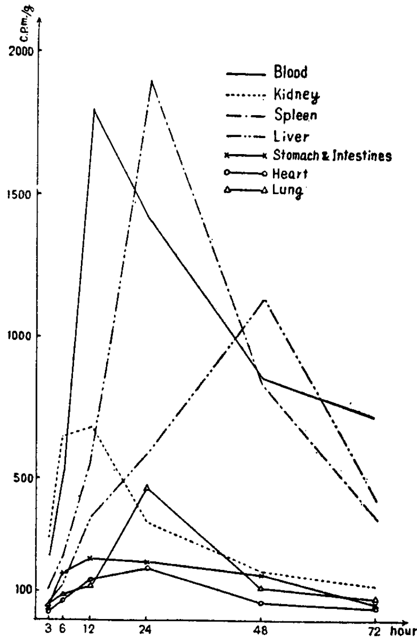


Fig. 3. (b)

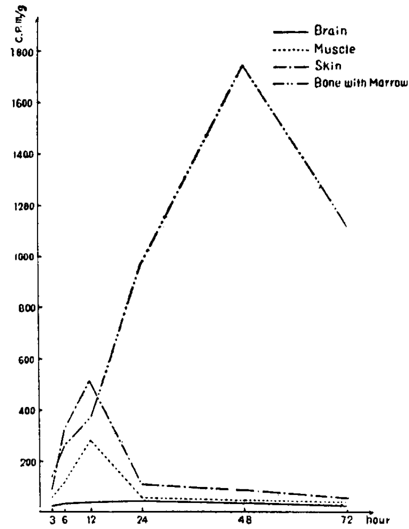


Fig. 3. (c)

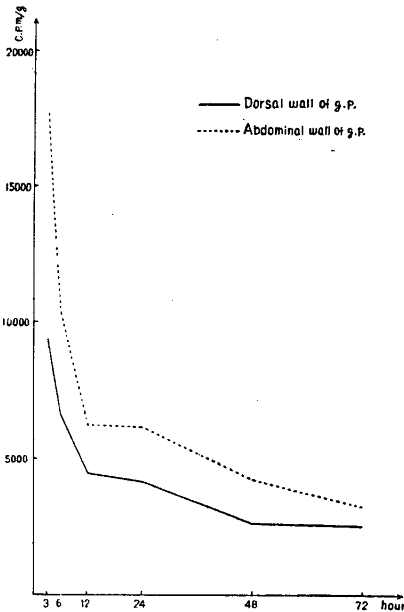


Fig. 3. (d)

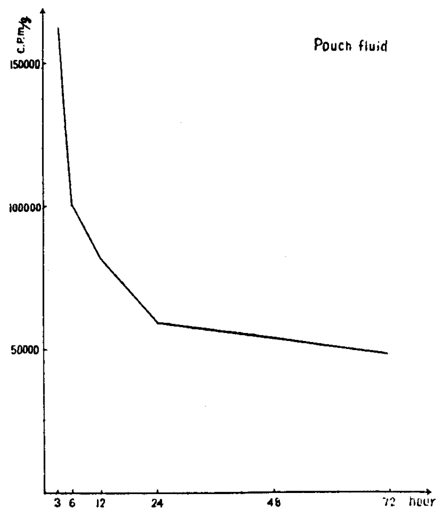


Fig. 3. Absorption and distribnution of ^{59}Fe labeled chondoritin sulfate-Fe from the rat granuloma pouch.

order. The abdominal wall of the granuloma pouch shows twice the radioactivity of the dorsal wall. The pouch fluid, approximately 5.5 ml, demonstrates a radioactivity of 895440.2 c. p. m. per ml. This is equivalent to 54.6 per cent of the total amount injected. Therefore, 45.4 per cent is presumably absorbed three hours after injection.

b) After six hours, the skin shows the third highest value in place of the bone marrow, and the stomach and intestines show a higher value than the liver.

c) After 12 hours, the increase of radioactivity of blood is marked, followed by the kidney, spleen and skin. Of the radioactive material injected into the granuloma pouch 72 per cent is absorbed.

d) After 24 hours, the radioactivity of the spleen is highest, followed by the blood and bone marrow.

e) After 48 hours, the bone marrow shows the highest radioactivity, followed by the liver, blood and spleen. On the other hand, the decrease in radioactivity of the kidney is marked.

f) At 72 hours, bone marrow still shows a high activity, with blood showing a comparatively high activity. The pouch still retains 16.2 per cent of the injected radioactive material.

4. *Summary*

a) Of the organs showing high values 12 hours after injection, that of the blood is markedly high with the increase being rapid. A relatively large amount of radioactivity is still seen 72 hours after injection.

b) At 24 hours, except for the pouch wall and pouch fluid, the spleen shows the highest radioactivity per gram tissue of all the organs. The peak radioactivity of the bone marrow is seen at 48 hours. These hematopoietic organs show different peaks, but the utilization, absorption and accumulation of Fe is large.

c) Absorption from the pouch is 45.4 per cent of the total amount injected three hours after, and 72.6 per cent 12 hours after injection.

d) Radioactivity of the skin was rather high 6 and 12 hours after injection in comparison to the other organs. However, contamination by urine and feces should be considered in this instance.

ABSORPTION AND ORGAN DISTRIBUTION OF ^{59}Fe LABELED FERRIC AMMONIUM CITRATE INJECTED INTO THE RAT GRANULOMA POUCH

1. *Materials and Methods*

As in the previous section ^{59}Fe labeled ferric ammonium citrate was injected into the granuloma pouch of rats and the organ radioactivity was measured at intervals. The radioactive material $^{59}FeCl_3$, was obtained from the Oak Ridge National Laboratory.

normality : 1.10 acid
^{59}Fe : 2.75±5 % mc/ml
specific activity of 59 Fe : 26190 mc/g
Fe$^{\cdot\cdot\cdot}$: 0.105 mg/ml
^{55}Fe : 0.040 mc/ml

As the toxicity of $FeCl_3$ is strong, carrier Fe was added in the form of ferric ammonium citrate to obtain ^{59}Fe labeled ferric ammonium citrate. Six groups of 4 rats each were injected approximately 5 mg of Fe, the ^{59}Fe being approximately 2.8 μc, 1.6×10^6 c. p. m. Radioactivity was measured by the same method mentioned in the previous section.

2) *Results and Summary*

The results obtained are shown in Fig. 4. To summarize the results :

a) At three hours after injection a large amount of radioactivity is already seen in the blood and bone marrow. The absorption from the granuloma pouch is 50.6 per cent of the total amount injected.

b) After six hours the increase in radioactivity of the spleen is markedly high, followed by the blood and bone marrow.

c) After 12 hours, except for the granuloma pouch wall and fluid, radioactivity is highest in the bone marrow showing the transportation of Fe to the

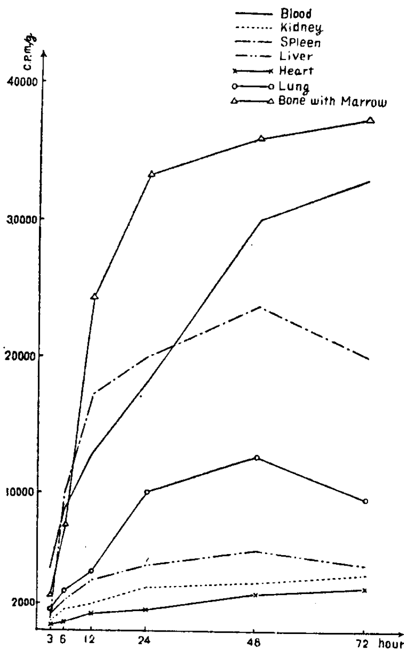


Fig. 4. (a)

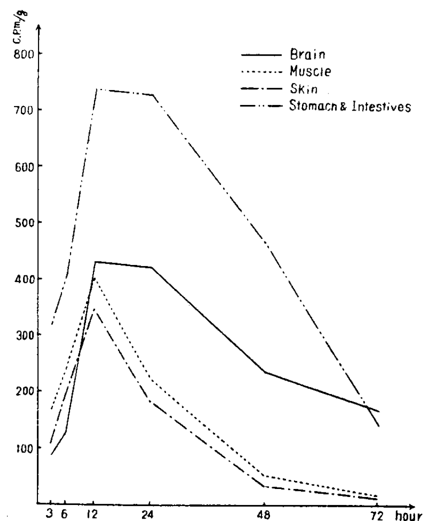


Fig. 4. (b)

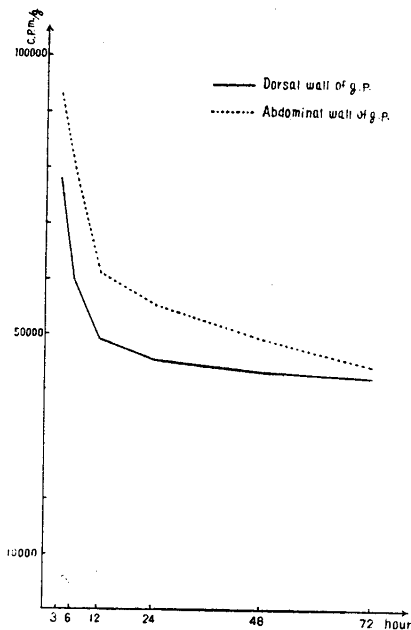


Fig. 4. (c)

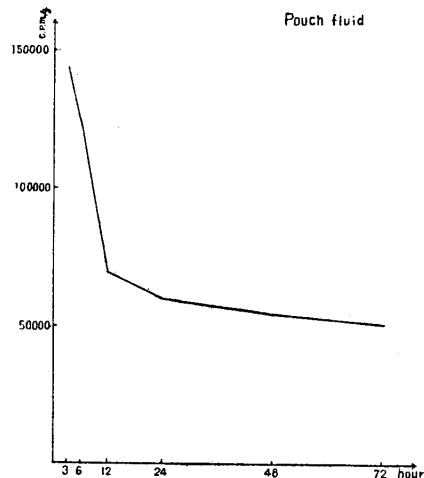


Fig. 4. (d)

Fig. 4. Absorption and disiribubution of ^{59}Fe labeled ferric ammonium citrate from the rat granuloma pouch.

hematopoietic organs. The maximum radioactivity is seen at 12 hours after injection in the brain, muscle, skin, stomach and intestines, subsequent decrease in activity.

d) At 24 hours radioactivity is strongest in the bone, spleen and blood in the same order. Although the spleen shows a peak activity at 48 hours and a subsequent decrease, the bone marrow and blood still show an increase.

e) From 48 to 72 hours, the bone marrow and blood still show an increase in radioactivity.

f) In Fig. 4, the lung shows a high radioactivity in comparison with the other organs. This is thought to be due to faulty exsanguination technic.

g) Injection of ferric ammonium citrate into the rat granuloma pouch and examining the absorption and organ distribution of Fe by time, showed a marked transportation of Fe to the hematopoietic tissue, especially the bone marrow.

ABSORPTION AND ORGAN DISTRIBUTION OF ^{59}Fe LABELED FERRIC AMMONIUM CITRATE AFTER INJECTION INTO THE RAT GLUTEAL MUSCLE

1. *Materials and Methods*

The ^{59}Fe labeled ferric ammonium citrate was prepared in the same manner set forth in the previous section. The labeled material, 1.5 ml per animal, was

injected into the right gluteal muscle of 24 male rats bearing a granuloma pouch formed according to the original method of Selye. The radioactivity of 1.5 ml of the ^{59}Fe labeled material was 1.6×10^6 c. p. m. The methods for measurement of radioactivity was identical to that given in the previous section.

2. *Results and Summary*

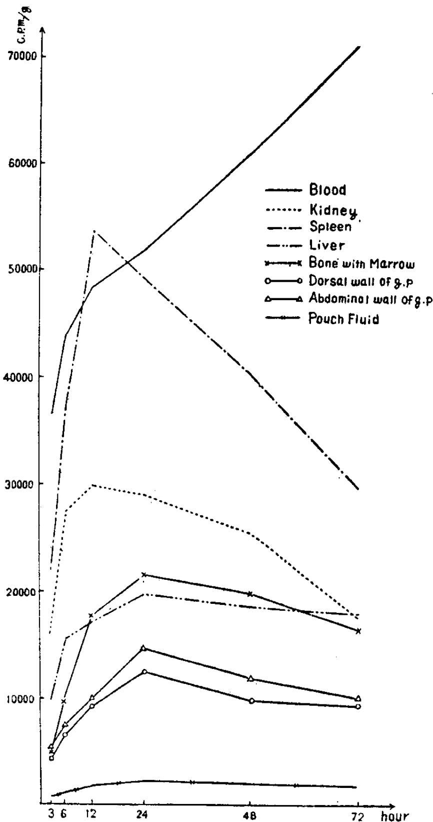


Fig. 5. (a)

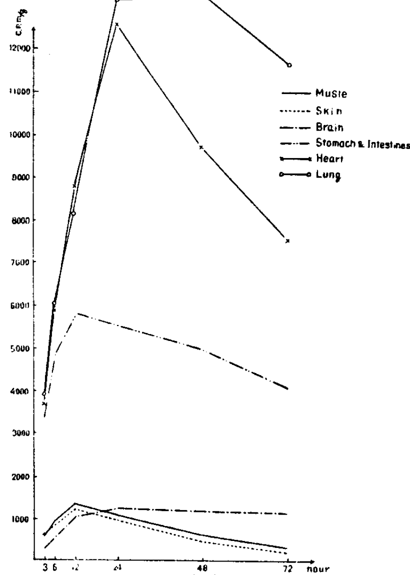


Fig. 5. (b)

Fig. 5. Absorption and distribution of ^{59}Fe labeled ferric ammonium citrate after injection into the rat gluteal muscl.

The results obtained are shown in Fig. 5. To summerize the results:

a) At three hours after intramuscular injection, the blood, spleen and kidney show a higher radioactivity than when the radioactive material is injected into the granuloma pouch. The bone marrow also reveals a high radioactivity, but its activity is lower than those of the afore-mentioned organs.

b) In each organ up to 12 hours after injection, the increase in radioactivity is markedly rapid in comparison with that seen in the case of intrapouch

injection. The radioactivity of the spleen decreasses thereafter in the case of intramuscular injection, whereas in the case where the material is injected into the pouch, the peak activity was seen 48 hours after injection. The radioactivity of the kidney is markedly increased over that seen in the previous section, with the slope of the curve being steep. The decrease after 12 hours is mild.

c) The curve of the radioactivity of blood becomes even steeper at 24 hours after injection, and can still be seen 72 hours after injection.

d) The radioactivity of the pouch fluid is very low in comparison with that of blood, the values being 1/30 to 1/40. The peak level was at 24 hours.

Radioactivity of the pouch wall is higher than that of the pouch fluid. Similar to the data shown in previous sections, the abdominal wall, in which development of the capillaries is more marked, shows a higher value than the dorsal wall.

e) Radioactivity of the bone marrow is higher than in the case of granuloma pouch administration three and six hours after the injection, but the ratio of increase is lower than that of the blood, spleen and kidney. Peak activity appears 12 hours after the injection, with a subsequent decrease.

ABSORPTION AND ORGAN DISTRIBUTION OF ^{198}Au LABELED GOLD COLLOID AFTER INJECTION INTO THE RAT GRANULOMA POUCH

1. *Materials and Methods*

As in the former experiment, male rats bearing 7 day granuloma were used. Inorganic gold colloid labeled with ^{198}Au was injected into the pouches, the animals were killed by exsanguination after different periods, 1 g of organ tissue was taken, and after homogenization the radioactivity was measured.

^{198}Au used in this experiment was obtained from the Radiochemical Center, Amersham, England and of the following chemical form.

sterilized colloidal suspension
stabilized with gelatin
concentration: 41.2 mc/ml
total solids: < 0.5 g/ml
pH 4—6

As the half life of ^{198}Au is only 2.71 days, the experimental procedure must be planned carefully. The radiation energy of ^{198}Au is 0.29 (1%) MeV. beta rays, 0.411 (100%) MeV. gamma rays.

The above ^{196}Au labeled gold colloidal suspension was diluted with physiological saline to a 0.2 per cent solution and 1 ml was injected into the granuloma pouch of each animal. The radioactivity was 6.99×10^6 c. p. m.

2. *Results and Summary*

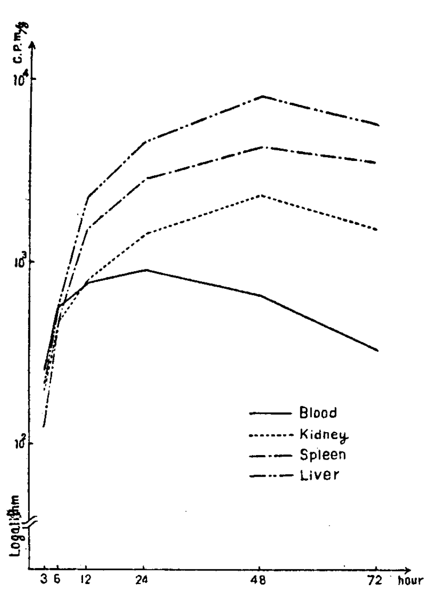


Fig. 6. (a)

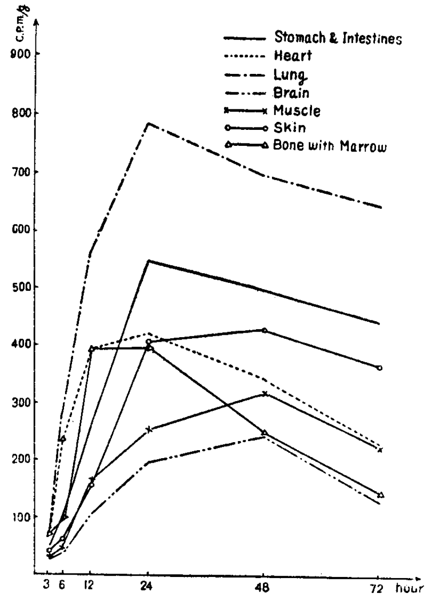


Fig. 6. (b)

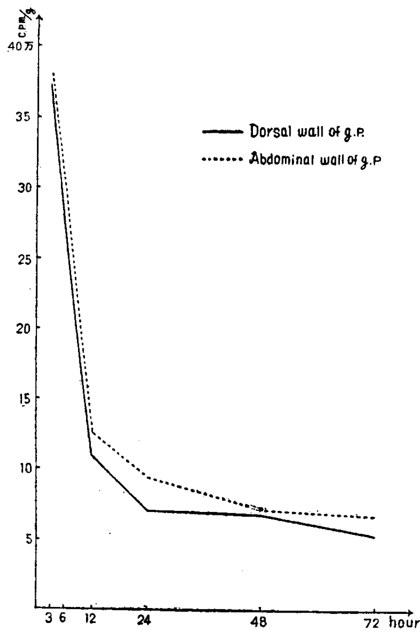


Fig. 6. (c)

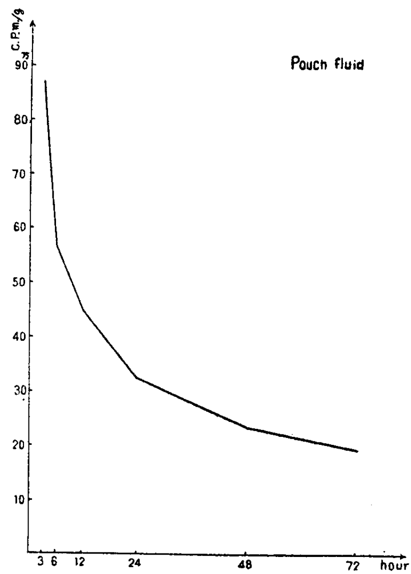


Fig. 6. (d)

Fig. 6. Adsorption and distribution of ^{198}Au labeled gold colloid from the rat granuloma pouch.

The results of the experiment, after being corrected for time, are shown in Fig. 6. The results can be summarized as follows:

a) The radioactivity of the blood shows a rapid increase three to six hours after injection, with a further gradual rise up to 24 hours. Thereafter it shows a gradual decrease, but the absolute values are lower and the rate of decrease larger than those of the liver, spleen and kidney.

b) The radioactivity of the spleen, liver and kidney is higher than that of the other organs, maximum values being seen 48 hours after injection.

c) The radioactivity of the blood and bone marrow is lower than that seen in the experiments with ^{59}Fe.

d) The abdominal wall of the pouch shows a slightly higher radioactivity than the dorsal wall.

e) The average amount of fluid in the pouch is 5.5 ml. Calculating the radioactivity remaining in the pouch from that of 1 ml of fluid, approximately 31 per cent of the radioactivity of the pouch is absorbed at three hours and approx. 74 per cent absorbed at 24 hours. Comparing this to the 50.6 per cent absorption at three hours in the case of ^{59}Fe labeled ferric ammonium citrate, the absorption of gold is slow.

From the above results, it is seen that the colloidal gold injected into the granuloma pouch has a tendency to be largely abosorbed by the RES organs.

ABSORPTION AND ORGAN DISTRIBUTION OF ^{198}Au LABELED GOLD COLLOID AFTER INJECTION INTO THE RAT GLUTEAL MUSCLE

1. *Materials and Methods*

The same ^{198}Au labeled gold colloidal suspension used in the previous section was used as the experimental material, 6.99×10^6 c. p. m. (1 ml of 0.2 per cent solution) being injected into the gluteal muscle of rats bearing a 7 day granuloma pouch. The animals were sacrificed at intervals and the radioactivity of the organ tissue measured with the scintillation counter.

2. *Experimental Results and Summary*

The organ distribution and absorption of ^{198}Au labeled gold colloidal suspension, when injected into the gluteal muscle, are as shown in Fig. 7.

a) Radioactivity of the blood shows maximal levels at three hours after injection, and thereafter it decreases.

b) The radioactivity of the liver is extremely high in comparison with those of the other organs, its peak being at 12 hours after injection.

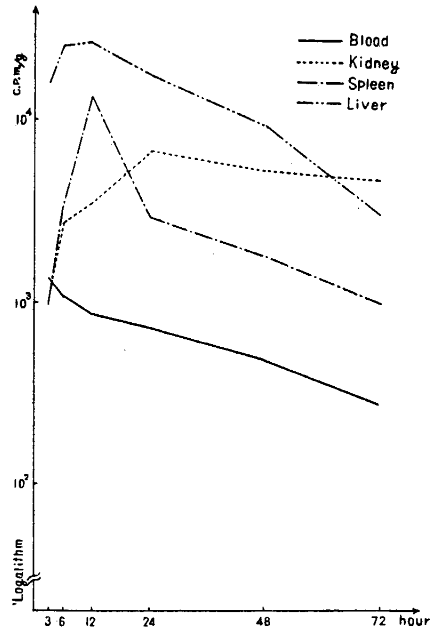
c) The highest value for the spleen is seen at 12 hours after injection, the value being second only to that of the liver. However, at 24 hours the radioactivity is lower than that of the kidney. At 72 hours, the radioactivity of the

kidney, although very low, is highest of all organs.

d) As seen in Fig. 7, the highest value for the kidney is seen at 24 hours, with a subsequent gradual decrease. This shows a continued excretion of gold in the urine.

e) Although no significant difference can be seen between the radioactivity of the abdominal and dorsal walls of the granuloma pouch, at 48 hours when maximum values are seen, the value of the abdominal wall is higher.

f) The maximum value of pouch fluid radioactivity appears after 24 hours. The value is very low compared to that of blood. However, the ratio



(Fig. 7. (a)

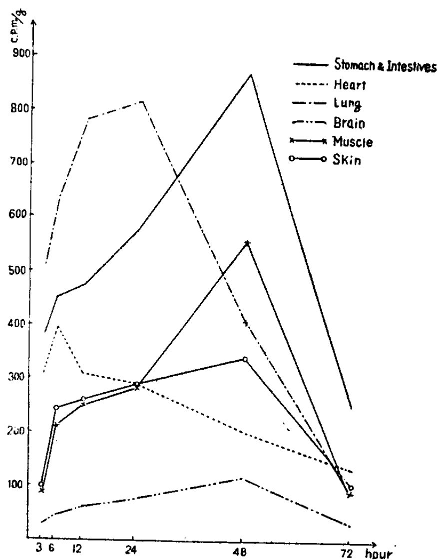


Fig. 7. (b)

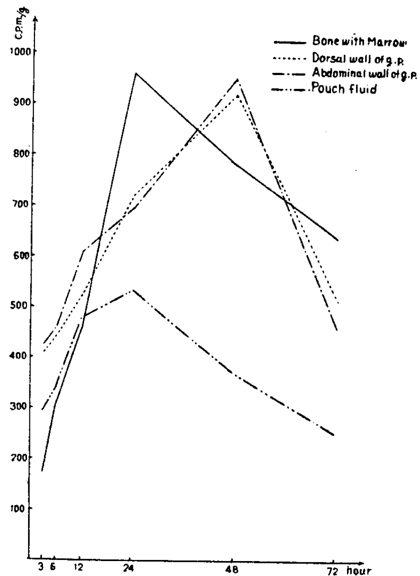


Fig. 7. (c)

Fig. 7. Absorption and distribution of ^{198}Au labeled gold colloid after injection into the rat gluteal muscle.

between pouch fluid and blood is markedly higher than the ratio in the case of when ^{59}Fe labeled ferric ammonium citrate is injected into the gluteal muscle.

DISCUSSION

The mechanism of fiber protein metabolism and non-collagen protein metabolism of connective tissue is still unknown at present. However, in regards to the metabolism of polysaccharides, LELOIR's[41)] sugar displacement reaction and LIPMANN's[42] sulfatizing reaction — Two great discoveries in the field of biochemistry — made it possible the development of the synthesis mechanism of HA and ChS by DORFMAN, SUZUKI[1], STROMINGER and LIPMANN.

Recently, WOLF has introduced Vitamin A[43] as the coenzyme of the mucopolysaccharide polymerization enzyme system, but the question is the metabolic turnover rate of the individual acid polysaccharide. The turnover rate of HA is clearly more rapid than that of ChS, and disturbances of this turnover rate causes important problems in clinical medicine. Such phenomena as maturation and aging cause a difference in the turnover rate[44], and also the effect of hormones must be important. The turnover of ^{14}C-acetic acid and ^{35}S to ChS is inhibited by cortisone and hydrocortisone[45]. On the other hand, pituitary growth hormone causes an increase in the metabolic turnover rate of ChS of bone and cartilage, but has no effect on the metabolism of HA[46]. In regards to ChS itself, the turnover rate of the isomers A, B and C perhaps differ, and thus the mechanisms should differ[47].

There are many reports that when ^{35}S is administered to animals the absorption is marked, especially in the bone and cartilage[30,48], and DZIEMIATKOWSKY has shown experimentally that the ^{35}S is mainly in the ChS of the bone and cartilage[48b]. BOSTRÖM injected ^{35}S labeled ChS intraperitoneally in rats and examined the ChS in the rib cartilage periodically. According to his report, the uptake and elimination of radioactive sulfate of the rib cartilage was seen for 16 days after injection, the maximum uptake being 24 hours after injection. Therefore, the extraction of ^{35}S labeled ChS in this experiment, as stated previously, was carried out 24 hours after the intraperitoneal injection of ^{35}S-sulfate.

The question of absorption from the joint cavity has been reported by KEY[49], ADKINS and DAVIES, BRAUN, BOHME, JAFFE, MAEDA, TAKIUCHI, INADOME, and many others. DOERING[50] injected ^{131}I into the joint cavity and reported that absorption is quicker in the presence of inflammation. Although nothing is known about the metabolism of steroid hormones in the joint, BLACK[51] has reported that steroids injected into joint ; (i) enter the circulation, (ii) are stored in the synovial tissue, and (iii) metabolized in the synovial tissue. WILSON[52] reported that 68 per cent of the hydrocortisone injected into the joint disappears from the synovial fluid within one hour, and BUNIM *et al.* reported that the half life of the free alcohol form is one to two hours, but hardly-soluble prednisolone trimethylacetate remains over a long period. HOLLANDER recommends predni-

solone t-butyl acetate. From these facts it can be supposed that less soluble esterisation of steroid hormones will raise their effectiveness in intra-articular injections. As the histological findings of the synovial membrane in rheumatoid arthritis and classification of disease types by KODAMA[55] and the studies of YOSHIHISA[56], ITO[57], etc., show that only a symptomatic significance can be expected from the present method of treatment of rheumatoid arthritis, the question of promotion of fibrosis of the synovial membrane arises.

In relation to these facts, the author injected various drugs into the joint cavity and examined their absorption. Further, from HAMMER's observation that the synovial membrane is nothing but connective tissue[58], the author has observed the metabolism of connective tissue. In this experiment, from various factors, SELYE's granuloma pouch was used.

In regards to ^{35}S labeled ChS, three hours after its injection into the granuloma pouch 43.2 per cent was absorbed from the pouch, the radioactivity of the kidney, liver, blood, heart, brain and muscle showed maximum levels 12 hours after injection, and the spleen, lungs, stomach and intestines, bone marrow and skin showed maximum levels 24 hours after injection. According to KISHIDA[59] who injected ^{35}S labeled ChS intraperitoneally into mature mice, the blood radioactivity showed maximum levels two hour after injection and in oral administration peak levels were reached six hours after administration. However, in the present experiment where the material was injected into the granuloma pouch, peak levels of the organs were reached in 12 to 24 hours, the time needed to attain peak levels being slower than in the case of intraperitoneal or oral administration.

Further, the radioactivity of the kidney was comparatively high, showing higher values than the other organs except for the bone marrow 72 hours after injection. The radioactivity of the urine was not examined, but according to KAPLAN[47] who injected the isomers A, B, and C of ChS intraveneously, ChS-A and C disappear from the serum within four hours after injection with only neglible amount being excreted in the urine, whereas about half the amount of ChS-B is excreted unchanged in the urine. As the ^{35}S labeled ChS used in this experiment was mainly ChS-A, only a small amount could have been excreted in the urine.

Radioactivity was also seen in the skin, muscle and bone marrow, among which the bone marrow showed the highest values. This indicates that the ChS entering the blood stream is gradually absorbed and fixed in the form ChS. This is in agreement with the data of DZIEMIATOKOWSKI, LAYTON, KISHIDA, etc.

There are many reports on the absorption and organ distribution of iron when administered to animals, and in recent years further development has

been seen by the used of radioactive iron[60]. The ChS-^{59}Fe and ^{59}Fe labeled ferric ammonium citrate injected into the granuloma pouch get into blood presumably via the lymphatic system, the $Fe^{\cdot\cdot}$ is changed into $Fe^{\cdot\cdot\cdot}$ by the action of oxidized hemoglobin, and conjugates with serum globulin, especially beta-globlin[61]. The iron carried to the various organs plays a role in the synthesis of hemogobin or the synthesis of cytochrome[62]. The metabolic course of iron in animals differs according to the method of administration and by the form of compound used.

When ChS-^{59}Fe is injected into the rat granuloma pouch, a high radioactivity is seen in the blood, spleen, liver and bone marrow, similarly as in the case of ^{35}S labeled ChS. Further, the fact that the decrease in blood concentration of radioactivity is larger than that seen in the case of when ^{59}Fe labeled ferric ammoninum citrate is injected in the granuloma pouch, and that the radioactivity of the spleen and liver is still high 72 hours after injection, means that the colloidal ChS-Fe is more rapidly transformed into iron fractions such as ferritin and hemosiderin than the inorganic iron compound, and that a larger amount is taken in by the reticuloendothelial system. This fact in regards to the utilization of iron in the body shows that the same tendency is seen in the case of granuloma pouch administration as to KOBAYASHI's experimental results[63] with the intravenous injection of a serum iron colloid, in which he reports that utilization of iron is highest when it is intravenously injected in the form of a colloid in which the iron ion is readily dissociated. Also, absorption from the pouch is slower in the case of ChS-^{59}F than ^{59}Fe labeled ferric ammonium citrate. This shows that the absorption of iron is slower when it is enveloped in the large ChS molecule than when it is in the form of an inorganic compound. From this fact, the absorption of steroid hormones injected in the joint cavity would be much slower when administered in an enveloped form, by a large molecular substance such as ChS, and that it would remain in the joint for a longer period.

Comparing the case where ^{59}Fe labeled ferric ammonium citrate was injected into the granuloma pouch with when it was injected into the gluteal muscle, in the former case the radioactivity of the bone marrow and blood still showed a tendency to increase 72 hours after the jnjection. It is presumed that when the iron concentration of the blood increases gradually, the marrow, spleen, etc., can utilize this sufficiently, but a rapid increase of blood concentration can not be coped with. This result is interesting in consideration of the report of FINCH and GRANICK[64] that in oral administration of iron the absorption stops when the blood metal-combining-protein is saturated. In practice, if such an administration method can raise the rate of iron utilizatoin of the body, this would be an interesting consideration in the administration of iron deficient patients.

In the administration colloidal ^{198}Au, high radioactivity was seen in the liver, spleen and kidney. In comparing this with the case of gluteal muscle

injection of colloidal ^{198}Au, the absorption was very slow with peak levels of the liver, spleen and kidney appearing 48 hours after injection. The blood showed the highest level 24 hours after injection and thereafter a decrease. Further, compared with the other material injected in the granuloma pouch, the radioactivity of the pouch walls was high in the case of colloidial ^{198}Au administration.

When colloidal ^{198}Au was injected into the gluteal muscle, a large amount of radioactivity was seen in the liver, spleen and kidney. Twelve hours after injection radioactivity of the liver, spleen and kidney was high, in that order; at 24 hours radioactivity of the kidney was higher than that of the spleen; and at 72 hours radioactivity of the kidney was higher than that of the liver. As OKAMOTO[66] has observed, distribution of gold in the body differs according to the method of administration, and also should differ with the type of gold compound used (this was seen in the case of iron adminstration in this experiment). OKAMOTO observed high radioactivity in the liver and spleen after intramuscular injection of colloidal ^{198}Au, and SUZUKI et al. reported a liver uptake of colloid ^{198}Au of 90.65 per cent in the dog. In the human, YAMASHITA et al. FELLINGER et al.[68] 78.2 per cent, and SAMUEL et al.[79] 60 to 94 per cent. On the other hand, HASHIMOTO[59] injected Aurotioglucose in the gluteal muscle of a rat (1 mg every other day, 6 injections) and reported the gold content of the kidney to be highest. Although radioactive gold is used in the treatment of various tumors[70], as reported in this experiment there is a difference by mode of administration colloidal, ^{198}Au is rapidly phargocytosed by the reticuloendothelial system and taken up by the liver and spleen, and also stored in the various other organs. Therefore, in the clinical use of this material attention must be paid to the general condition of the body.

In the injection of colloidal ^{198}Au into the gluteal muscle, radioactivity of granuloma pouch fluid was always lower than that of blood. This agrees well with the findings of HASHIMOTO in his Aurotioglucose experiments. However, in the case of ^{59}Fe injection into the gluteal muscle the difference between radioactivity of pouch fluid and blood was great, but in the case of ^{198}Au the difference was not so great. Further, with ^{59}Fe and ^{198}Au in both intramuscular injection and intra-pouch injection, radioactivity of the abdominal wall of the pouch was higher than that of the dorsal wall.

In the present experiment the organ absorption and distribution of $Ch^{35}S$, ChS-^{59}Fe, ^{59}Fe and ^{198}Au were examined after their injection into the granuloma pouch and into the gluteal muscle. At present, microradioautographic experiments are being carried out to examine how these materials are taken up and by what tissue of various organs. It is hoped that these experiments will throw some light on the metabolism of antirheumatic agents when injected into the joint cavity in rheumatoid arthritis.

SUMMARY

A granuloma pouch was formed on the back of rats by the original method of SELYE. Seven days when granuloma tissue reached its maximum, ^{35}S labeled ChS, ^{59}Fe labeled ChS-Fe, labeled ferric ammoninum citrate and colloidal ^{198}Au were injected into the pouch and their absorption and organ distribution examined and compared with the results in the case where ^{59}Fe labeled ferric ammoninum citrate and colloidal ^{198}Au were injected into the gluteal muscle.

1. When ^{35}S labeled ChS was injected into the granuloma pouch, radioactivity of the organs per gram tissue was high in the kidney, liver, bone marrow and spleen, in descending order. The maximum activity was seen 12 to 24 hours after injection, which is slow compared to the results obtained by KISHIDA in intraperitoneal and oral administration.

2. The absorption of Ch S-Fe by pouch where the iron is enveloped by the large ChS molecule, is slower than that of ferric ammonium citrate, an inorganic compound.

3. The uptake of Fe from the blood by bone marrow is larger when the increase of blood Fe ion concentration is slow, rater than when the increase is rapid.

4. When colloidal ^{198}Au is injected into the pouch and injected into the gluteal muscle, the ^{198}Au is phargocytozed by the reticuloendothelial system organs, the liver showing the largest uptake among all organs.

5. In the intramuscular injection of colloidal ^{198}Au and ^{59}Fe labeled ferric ammonium citrate, radioactivity of pouch fluid is lower than that of blood. However, the difference between the two is less in the case of colloidal ^{198}Au.

6. In the granuloma ponch, radioactivity of the abdominal wall proves to be greater than that of the dorsal wall.

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