HISTOCHEMISTRY OF CATECHOLAMINE

Mainly concerning with the spleen

by

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Received for publication Feb. 3, 1962

Among various organs, the amount of catecholamine in the spleen is next to that of the adrenal medulla, as it has been known by the studies of EULER et al.[7)8)11)] In addition, EULER [14)] proved chemically that splenic nerve contains "noradrenaline". EULER and HILLARP[12)] showed that noradrenaline was present in the submicroscopic particles from the homogeniz ed splenic nerves.

In this study, histochemical and electronmicroscopic findings of catecholamine-like substance which is abundantly distributed in the spleen are reported.

(A) HISTOCHEMICAL OBSERVATION

Using the classical chromaffin reaction of HENLE,[48)] the author used the partially modified HILLARP and HOKFELT's method.[21)22)31)32)33)34)35)36)]
Fluorescence method was also used.[15)16)17)18)19)69)]

(I) Experimental materials and methods

(a) Adrenal medullae, spleens and livers of normal or splenomegalic patients and rabbits were used.

(b) Staining methods

Following three methods of staining were used.

* Method 1 Bichromate-chromate method [9)32)33)34)35)36)56)]

Fixing fluid : 5% Potassium bichromate 10 volumes
5% Potassium chromate 1 volume
Fixing time : 48 hours
Embedded in Paraffine
Counterstain : Hematoxylin or Kernechtrot
Mounted in Canada balsam

With this method the cells in the adrenal medulla, which contain noradrenaline are stained in yellow to yellowish-brown and the cells which contain adrenaline are stained in dark brown.

* Method 2 Iodate method[20)32)33)35)36)]

The fixing fluid is made of 10% potassium iodate solution with pH 5.4 phosphate buffer or pH 6.0 acetic acid acetate buffer.

The fixing time, paraffine embeding and counterstain are same as the method 1.

With this method the cells in the adrenal medulla which contain noradrenaline are selectively stained in yellowish brown.

* Method 3 Fluorescence method[10)15)15)17)18)19)49)50)60)66)67)69)]

Fixing fluid : 2% Calcium chloride 5 volumes
Distilled water 4 volumes
Neutral formaline 1 volume
(kept with $CaCO_3$)

Fixing time : 24 hours

Blocks were made by dry ice acetone method and they were cut by Cryostat[33)] and mounted in glycerin.[43)]

Fluorescence microscope was used consisting of Osram HBO 200 ultrahigh pressure mercurial light, illumination apparatus and standard microscope of Carl Zeiss with filter, BG 12, KG 1 and OG 5.[2)27)69)]

With this method, the tissue which contains catecholamine in the adrenal medulla is observed as fluorescing islet. The fact that catecholamine in the tissue gives specific fluorescence has already been confirmed.

(Ⅱ) Animal experiments and the results

(a) Development of splenomegaly by albumin-sensitization[52)61)]

Using 30 white rabbits, chiken egg albumin was injected intravenously daily for 3 months in 25 of them and the others were left untreated as controls.

Splenomegaly was developed in all sensitized rabbits 3 months after the first albumin injection.

In normal rabbits spleens weighed less than 1 g while in the sensitized rabbits 2.10 g-6.65 g This is shown in Fig. 1.

(b) Findings in the adrenal medulla

The findings were same as above described both with bichromate-chromate and iodate methods. There was no significant difference even in the sensitized splenomegalic rabbits. These are shown in Fig.2 and 3.

By fluorescence method, the same was true and shown in Fig.4.

(c) Findings in the spleen

(i) In the spleen of normal rabbits

Among the splenic trabecular cells, those which had yellow cell-substance were rarely observed. Fig. 5 shows a picture with bichromate-chromate method. Fig. 6 is with iodate method.

The cells with yellow or yellowish brown cell-substance were observed in the red splenic pulp with iodate method, as is shown in Fig. 7. The same was observed in bichromate-chromate method.

(ii) In the spleen of sensitized splenomegalic rabbits

In and around the splenic trabecula, the cells with yellow cell-substance were frequently observed. This is shown in Fig. 8.

The cells with yellow or yellowish brown granules within them were observed in the red splenic pulp, as is shown in Fig. 9 and 10, while in the white splenic pulp there was no such a cell.

(d) Findings in the liver

(i) In the liver of normal rabbits

There was no cell with yellow cell-substance (Fig. 11).

(ii) In the liver of sensitized splenomegalic rabbits

The cells facing the interlobular connective tissue were rarely stained yellowish brown. This is shown in Fig. 12.

These cells in Fig. 13 were not distinguishable from phagocytes.

(Ⅲ) Clinical observation

The following 17 cases were also studied.

BANTI's syndrome	12 cases
Familial hemolytic jaundice	1 case
Patients with normal spleen	4 cases (3 Gastric ulcers and 1 CROHN's disease)

(a) Normal spleen of the patients

(i) Findings in the spleen

As shown in Fig. 14, the cells with yellow chromaffin reaction were very rarely observed. With fluorescence method, autofluorescence was observed in the travecula and in the surroundings of the sinus in the red splenic pulp, as is shown in Fig. 16.

(ii) Findings in the liver

No cells with chromaffin reaction were observed in the normal liver, except for unknown fine granules within the parenchymal cells (Fig. 15).

With fluorescence method the liver cells had some fluorescence granules especially in those surrounding the central vein (Fig. 17).

(b) BANTI's syndromes

(i) Findings in the spleen

Relatively large cells with positive chromaffin reaction were observed in the red splenic pulp, as is shown in Fig. 18, while none in the white splenic pulp.

They gave no difference from the normal spleen with fluorescence method. However, in this disease, there was marked increase of connective tissue, which might have a fluorescing nature. No fluorescence was seen in the white splenic pulp.

(ii) Findings in the liver

As shown in Fig. 19, there were cells with yellow cell-substance which was considered to be due to positive chromaffin reaction. There was no definite difference from normal liver even with fluorescence method.

(c) Familial hemolytic jaundice

Brownish granular pigments were observed not only in the spleen but also in the liver. The presence of jaundice possibly gave the findings, which was hard to evaluate (Fig. 20, 21).

(Ⅳ) Histochemistry of monoamine-oxidase[4)25)29)32)]

Many histochemical methods were proposed to study monoamine-oxidase, which inactivated catecholamine. In the present study, potassium tellurite reduction method of TAKAMATSU was used. The spleen and liver which were stained with this method are shown in Fig. 22 and Fig. 23.

There was no definite correlation between normal and splenomegalic spleens.

(V) Discussion and summary

(a) The author observed the cell-substance which showed positive chromaffin reaction, in the normal and splenomegalic spleens.

EULER et al[78][11] identified noradrenaline from all organs innervated with adrenergic nerve. EULER and HILLARP[12] showed that noradrenaline was present in the submicroscopic particles from homogenized splenic nerves. EULER[14] substantiated noradrenaline in the splenic nerve by chemical assay. HILLARP and HÖKFELT[33] observed that purified adrenaline and noradrenaline showed positive chromaffin reaction. With this previous observation, it is very probable that certain cells in the spleen contain noradrenaline and would give positive chromaffin reaction.

Concerning the staining, LISON et al[48] claimed that histochemical observation could identify adrenaline only in the adrenal medulla and paraganglion.

UONO et al[65][66] reported that in the splenic and hepatic parenchyma there were cells which had yellow or yellowish brown cell-substance in chromaffin reaction. Considering the reports,[35][1] in which a non-chromaffin type of pheochromocytoma and paraganglioma was described, we should reexamine the relation between catecholamine and chromaffin substance.

(b) Splenic pigments[28][30]

MÖLLENDORF[28] described that hemosiderin, erythrophagocytes and other iron components showed yellow or yellowish brown colour. The author could not differentiate the iron components from the positive chromaffin substance, as they were almost identical in colour. Therefore the author tried to differentiate the iron components from the chromaffin substance with the morphological method. According to LISON et al,[4][26] iron staining reagents cannot be used for the differentiation of iron from the chromaffin substance, because they are reduced by chrome as well as iron, when chrome is contained in the fixing fluid.

(c) Many cells which had positive chromaffin reaction were observed in the spleen of splenomegalic patients.

FUJII[23] reported that myelinated fibres of the splenic hilum were increased in BANTI's syndrome. This is well understood when above mentioned reports by EULER,[14] EULER and HILLARP[12] are taken into consideration.

(d) According to SUGITANI,[40][60] catecholamine in the portal blood did not necessarily increased in BANTI's syndrome. Contradiction between the present histochemical findings showing increased catecholamine in the splenomegalic spleen and SUGITANI's[40][30] finding needs further investigation.[13][39][40][41][30]

(e) Fluorescence method[2][27][69]

In the author's study, the white splenic pulp had neither fluorescing ability nor chromaffin reaction. The autofluorescence is generally weak and lacks specificity, so it is not suitable for observation.[2][69] Especially in the liver Vitamin A also shows strong fluorescence, which disturbs the observation of catecholamine.[69]

(f) Concerning monoamine-oxidase, no constant result was obtained by histochemical method,[37][38][39] though there were some questions in this staining method of TAKAMATSU.[32]

This can be understood from the fact, as AXELROD[1] reported, that not only monoamine oxidase but also catechol-O-transferase are related to the metabolism of catecholamine.

(B) OBSERVATION BY ELECTRON MICROSCOPE

The chromaffin substance of the adrenal medulla has affinity with osmic acid, as was proved by COUJARD and COUJARD-CHAMPY.[48] However, the substance with osmium affinity in the adrenal medulla was not always catecholamine or chromaffin substance. It was already confirmed by LEVER,[44)46)46)47)] DE ROBERTIS and VAS FERREIRA,[53)54)55)] FUJITA[24)] and others that electron dense granules were present in the cells of the adrenal medulla.

The following study was undertaken to investigate the catecholamine-like substance and the cells containing it in the spleen and liver.

(Ⅰ) Experimental materials and methods

Purified noradrenaline, adrenaline and microcrystals of colloidal gold were used as the controls and white rabbits as the experimental animals.

The materials were fixed in equal volume of DALTON Buffer and 4% osmic acid solution for 2 hours and then dehydrated, infiltrated in propylen oxide and embedded in Epon (Epon 815 : Epon 812=1:1).[64)] Used electron microscope were Hitachi Type HS-6, HU-10 and HU-11.

(Ⅱ) Experiment I and the results

Similarity between splenic and adrenal cells in the rabbits was investigated.

Electron micrograph of the spleen of normal rabbits is shown in Fig. 24. There are cells with mitochondria and electron dense granules. These cells are frequently observed at the red splenic pulp. The cells of adrenal medulla of normal rabbits are shown in Fig. 25.[34)] These are many electron dense granules in the cell substance

(Ⅲ) Experiment II and the results

(a) Purified noradrenaline and adrenaline solution were fixed, embedded, sectioned and were observed by electron microscope. They showed electron dense granular picture.

(b) Microcrystals of colloidal gold were prepared by SUITO's method[57)58)59)] and was observed by electron microscope. Its microdiffraction pattern was observed and shown in Fig. 26.

(Ⅳ) Experiment III and the results

3 ml. (5mg/ml.) of above mentioned microcrystals of colloidal gold and 20% glucose solution were mixed and injected into auricular vein of rabbits daily for 5 days. After 5 days, the rabbits were anesthetized by pentobarbital (Nembutal) and were sacrificed. The sections of spleen and liver were prepared and observed by electron microscope.

(a) Findings in the spleen

As shown in the lower central part of Fig. 27, phagocytes contained mitochondria and regular or irregular shaped electron dense granules in the cell-substance. The microdiffraction pattern of this regular shaped electron dense granules is shown in Fig. 28 and Fig. 30. This diffraction pattern was analized and was found to identical as that of gold. A phagocyte is shown in the lower central part of Fig. 29, which contains mitochondria and takes up two erythrocytes.

One of them is disfigured and both of them are surrounded by limiting membranes. There is also the structure looks like myeline. Fig. 31 shows the electron dense granules in the splenic cell. The dark and light parts are intermingled. Besides the phagocytes, many cells containing electron dense granules and mitochondria were often observed.

(b) Findings in the liver

Fig. 33 shows the microcrystals of colloidal gold contained in a liver cell.

Fig. 32 is its microdiffraction pattern by electron microscope.

(V) Discussion and summary

(a) Identification of microcrystal of colloidal gold and phagocytes in vivo.

As the author previously reported at Kansai Meeting of Electron Microscope Society, the part of the electron dense granules in the cells are microcrystals of colloidal gold. This fact is proved by satisfying BRAGG's condition and diffraction constant "d" from the diffraction pattern. The cells with the granules which showed diffraction pattern of gold should be phagocytes. The administration study of colloidal gold can be regarded as similar to KIYONO's vital staining method.[42]

(b) Catecholamine-like substance and electron dense granules in the spleen.[5)38)38)]

As described by COUJARD and COUJARD-CHAMPY,[48] the chromaffin substance had osmium affinity. Catecholamine, a part of chromaffin substance, was also proved to have electron density by the present study. Though many investigators reported that the electron dense granules with osmium affinity in the adrenal medullary cells are related to catecholamine, they did not directly proved it. There are some splenic cells which contain electron dense granules.

Since gold was not shown in these cells by the administration study of colloidal gold, these are not regarded to be phagocytes. These electron dense granules with osmium affinity are morphologically similar to secretion granules in the adrenal medulla.

ELFVIN[7] assumed experimentally that there was close relationship between the granular structure which was observed within the splenic nerve and catecholamine.

According to EULER,[14] EULER AND HILLARP,[12] noradrenaline was identified in the splenic nerve fraction, especially in the submicroscopic particles.

According to EULER et al,[11] the amount of catecholamine in the spleen is larger than that in the splenic nerve. Thus catecholamine should be contained in the spleen besides the splenic nerve. By the experimental study of SUGITANI[10] there should be some part within the spleen where is especially related to catecholamine.

The author can not help considering the close relationship between the electron dense granules observed in the certain splenic cells and catecholamine, because the close resemblance in the shape and density were observed between them and the electron dense granules were present in the splenic cells other than phagocytes.

(C) CONCLUSION

(1) In the spleen and the liver, cells which had chromaffin affinity were observed.

(2) In the spleen of sensitized splenomegalic rabbits with albumin, the cells with chromaffin substance were relatively well observed.

(3) In the spleen of BANTI's syndromes the cells with chromaffin substance were also observed more abundantly than the normal spleen in the histochemical study.

(4) In the phagocytes of rabbits which were injected microcrystals of colloidal gold intravenously, two kinds of electron dense granules were observed. The one showed regular shape with the diffraction pattern of gold and the other irregular shape without

the same diffraction pattern.

(5) In the certain cells of the spleen other than phagocytes, many electron dense granules were also observed, having close relation morphologically to catecholamine-like substance.

The author would like to express his gratitude to Assistant Professor Ch. Kimura who gave the constant help and good suggestion throughout this work.

The author is very grateful to Dr. Y. Hamazima, Assistant Professor of Pathology, for his helpfulness in the investigation concerning the fluoresence method.

The author wishes to thank Dr. K. Tanaka, Professor of Physics, for encouragement throughout the course concerning electron diffraction study and to thank Dr. Hiroshige, director of Shinko Hospital at Kobe, and Dr. Hashino for their helpfulness about the use of electronmicroscope Hitachi HU-10.

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和文抄録

カテコールアミンの組織化学的研究

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脾臓は大部分アドレナリン性神経に支配される唯一の臓器であり，且つ教室杉谷によれば，門脈のHomeostasis に向つての役割を演じている．更に Hillarp, Hökfelt 及び Eränkö 等に依れば，副腎髄質に於ては，アドレナリン及びノルアドレナリンが夫々特定の細胞に含有されているという．即ち本研究に於ては，脾臓に於ても，かかる特殊の細胞の存在を組織化学的及び電子顕微鏡的に識別し得るか否かを実験に匡し，依つて脾臓の神経性調節機構の一端をうかがうものである．

観察方法は，Hillarp及びHökfeltのクロム親和反応を応用し，副腎に於ては，アドレナリン及びノルアドレナリンが，同時に，しかも区別されて染色されるという，重クロム酸クロム酸法と，ノルアドレナリンのみが選択的に染色されるという，沃素酸カリ法を使用して，更に Eränkö による螢光法及び電子顕微鏡による観察法をも応用した．

脾臓に対して，組織化学的方法を使用することの可

否に就いても充分に考慮し，またカテコールアミンの分解酵素の一つである，アミンオキシダーゼの組織化学的検索には高松法を使用した．

主な対象は，正常及び脾腫を呈した人間並びに家兎の脾臓である．この脾腫家兎作製には，主としてアルブミン感作法を応用，また電子顕微鏡による観察を行う家兎には，コロイド状金薄片粒子を静注して貪喰細胞の貪喰にまかせた．

以上の実験から，次の結論を得た．

1) 稀ではあるが，健常の脾臓及び肝臓に，クロム親和反応に際して，黄色を呈する細胞質を持つ細胞の存在することが観察された．

2) ところが，アルブミン感作脾腫家兎の脾臓にはクロム親和反応に際して，この黄色の細胞質を有する細胞が，健常家兎よりも多数に存在することが，組織化学的方法によつて立証された．

3) また，バンチ氏病脾臓に於ても，クロム親和反応に際して，黄色細胞質を有する細胞が，健常人脾臓に於けるよりも多数に存在することを，組織化学的に立証した．

4) 而もかかるクロム親和反応に際して，黄色細胞質を有する細胞の観察されるのは，ほとんど凡て，赤色脾臓の範囲内である．

5) 更にコロイド状金薄片粒子を家兎静脈内へ注射して，その脾臓を電子顕微鏡的に検査すると，金コロイド粒子を貪喰しているとかかる貪喰細胞ではない別の或種の細胞に，高電子密度顆粒が多数に含まれていることが観察されたが，後者に含まれている高電子密度顆粒は，副腎髄質等に含まれているカテコールアミン様顆粒と形態的に類似しているから，恐らくカテコールアミン様物質と密接な関係があるものと思われる．

6) この際の貪喰細胞は，貪喰された金コロイド粒子に対する，電子線顕微回折法によつて除外することが出来るものである．

7) なお，Eränkö の螢光法を以てしては，白色脾髄には螢光を証明しがたく，赤色脾髄に螢光が観察出来たという以外，特別の結論は得られなかつた．

8) アミンオキシダーゼの組織化学的検索に於ても，一定の結論は得られなかつた．このことに関しては，アミンオキシダーゼ以外にも，カテコールアミン分解酵素として，カテコール—O—メチルトランスフォレースがあることを考え併せれば理解出来ないことはない．なお，此の酵素に対する組織化学的検索は未だ確立されていないものである．

Fig. 1 Spleens of rabbits

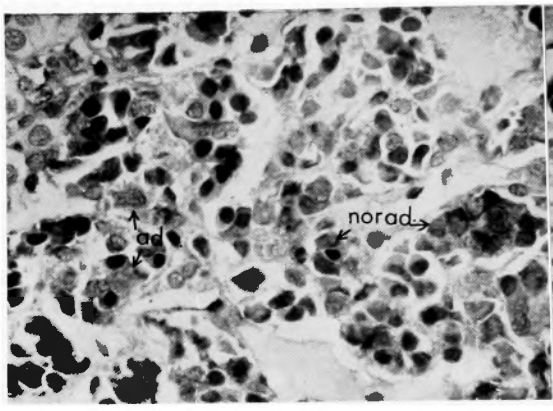

Fig. 2 Adrenal medulla 40×10
Bichromate-chromate method

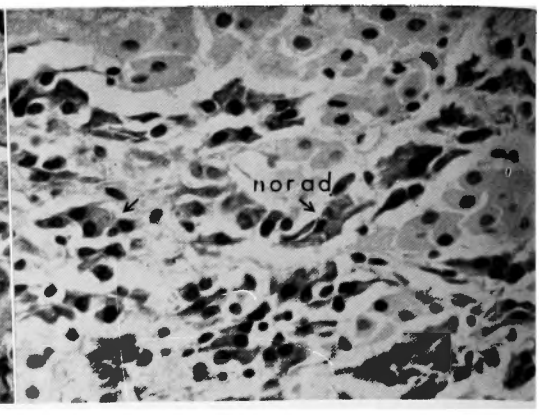

Fig. 3 Adrenal medulla 40×10
Iodate method

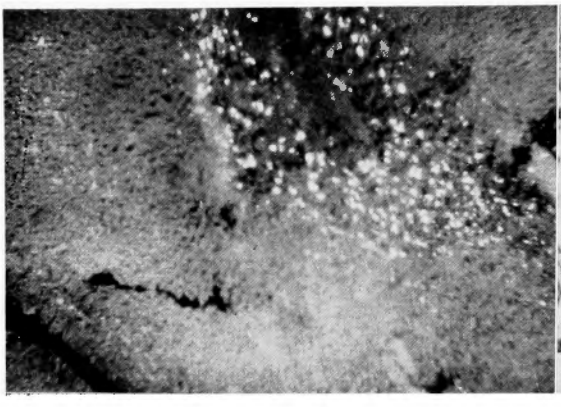

Fig. 4 Adrenal medulla 10×10
Fluorescence method

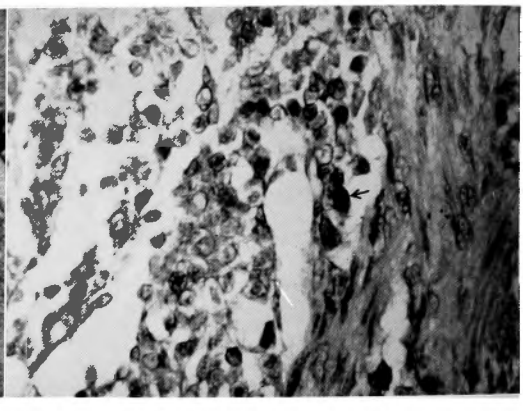

Fig. 5 Spleen of normal rabbit 40×10
Bichromate-chromate method

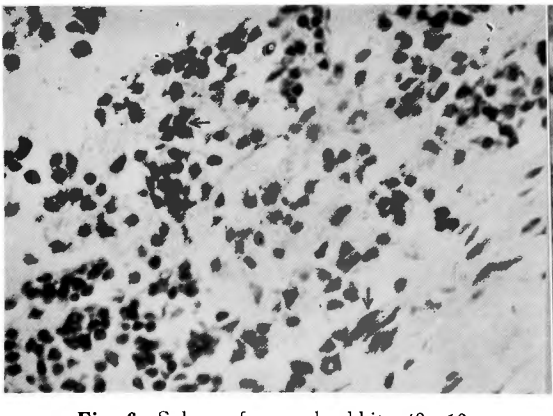

Fig. 6 Spleen of normal rabbit 40×10
Iodate method

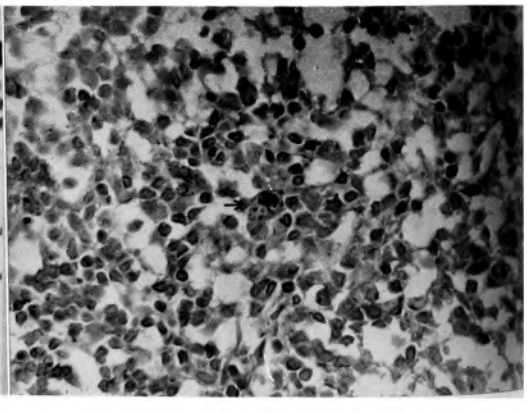

Fig. 7 Spleen of normal rabbit 40×10
Iodate method

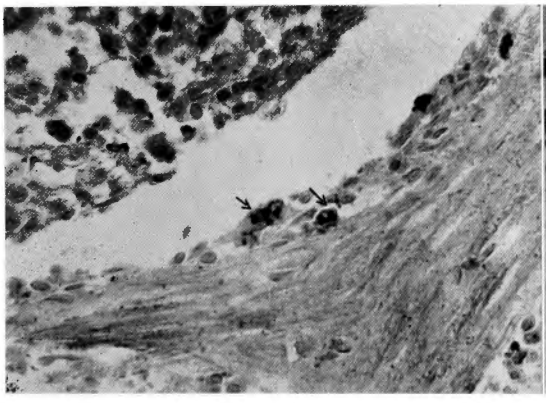

Fig. 8 Spleen of sensitized rabbit 40×10
Iodate method

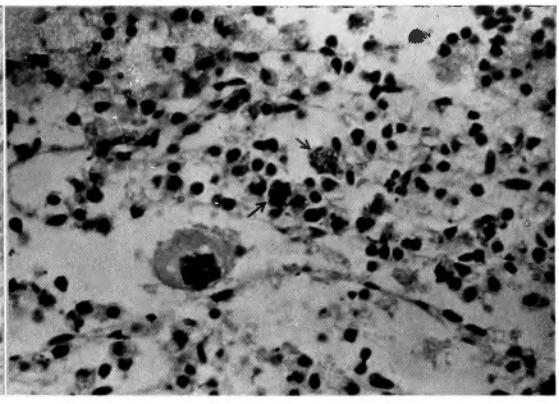

Fig. 9 Spleen of sensitized rabbit 40×10
Iodate method

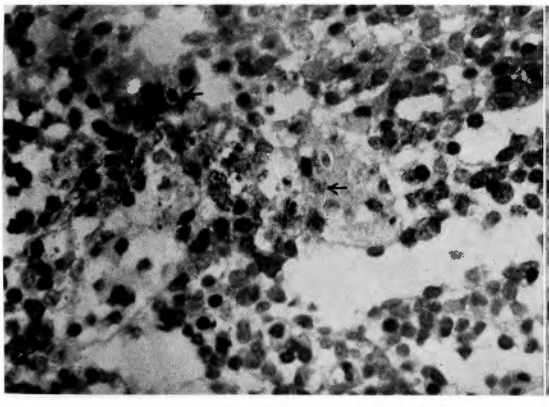

Fig. 10 Spleen of sensitized rabbit 40×10
Bichromate-chromate method

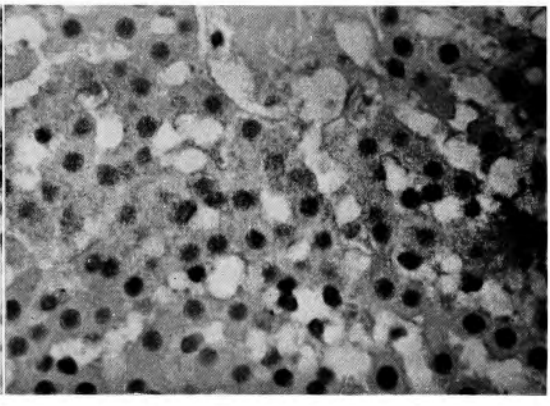

Fig. 11 Liver of normal rabbit 40×10
Bichromate-chromate method

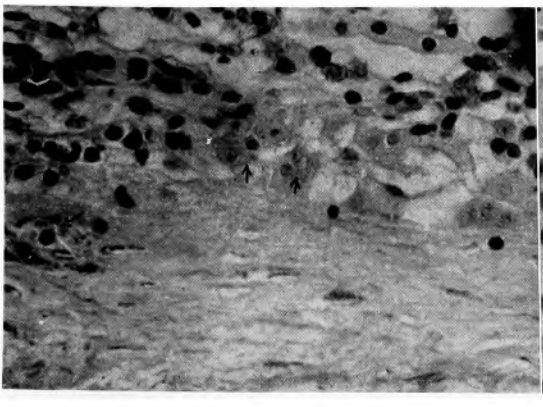

Fig. 12 Liver of sensitized rabbit 40×10
Bichromate-chromate method

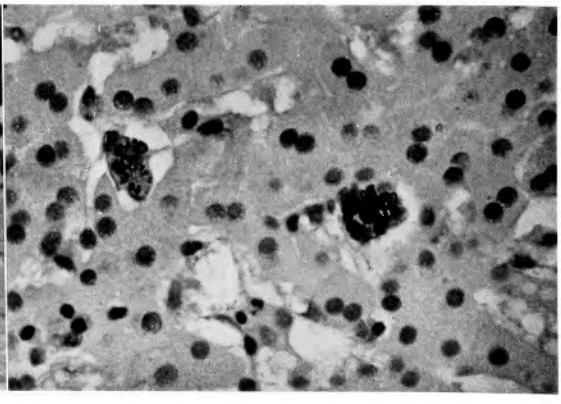

Fig. 13 Liver of sensitized rabbit 40×10
Bichromate-chromate method

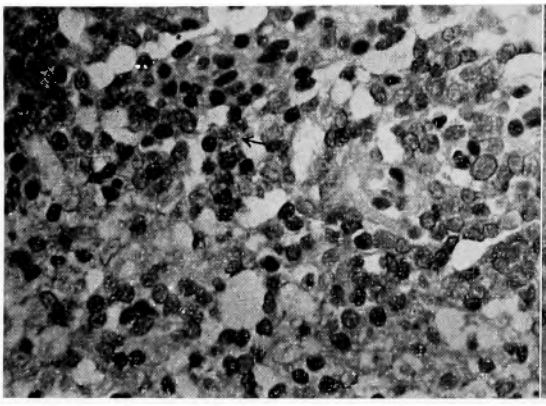

Fig. 14 Normal spleen of a patient 40×10
Bichromate-chromate method

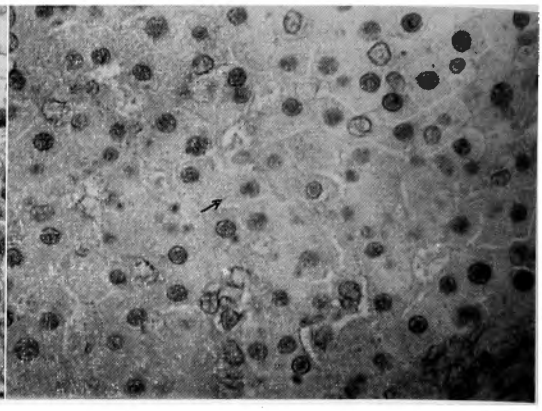

Fig. 15 Normal liver of a patient 40×10
Bichromate-chromate method

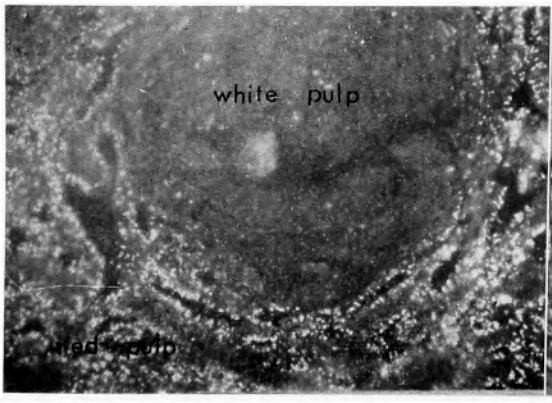

Fig .16 Normal spleen of a patient 10×10
Fluorescence method

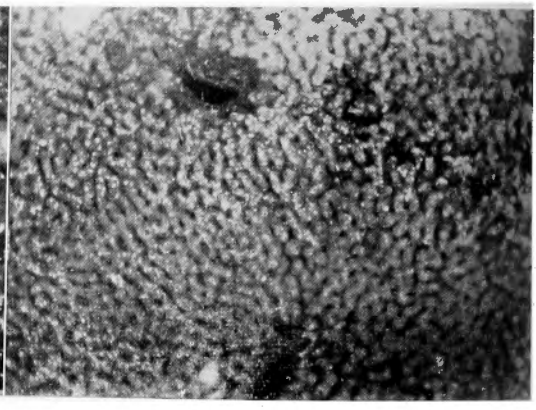

Fig. 17 Normal liver of a patient 10×10
Fluorescence method

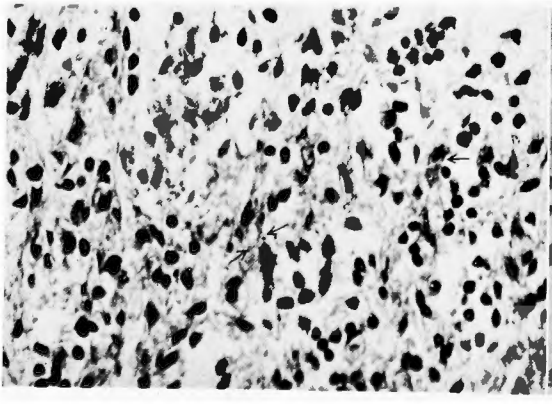

Fig. 18 Spleen of Banti's syndrome 40×10
Iodate method

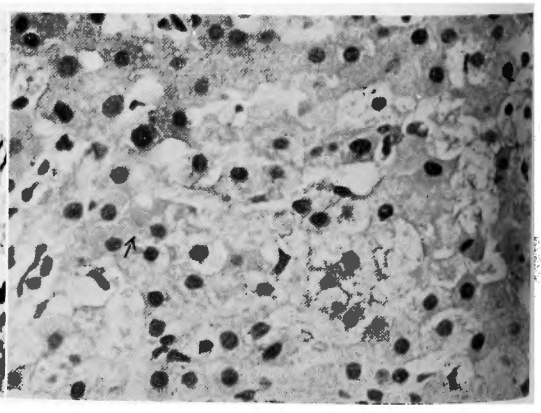

Fig 19 Liver of Banti's syndrome 40×10
Iodate method

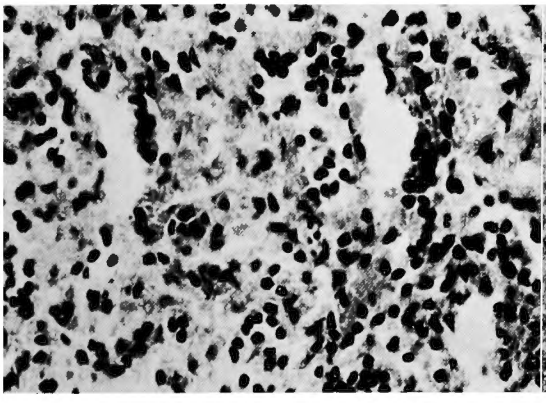

Fig. 20 Spleen of familial hemolytic jaundice
Bichromate-chromate method 40×10

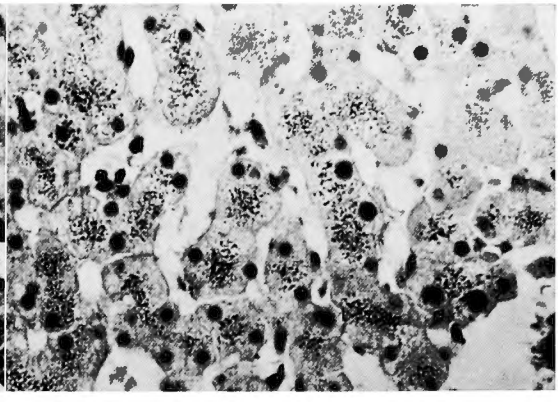

Fig. 21 Liver of familial hemolytic jaundice
Iodate method 40×10

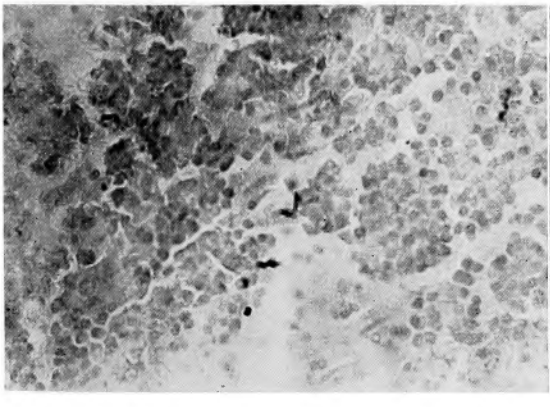

Fig. 22 Spleen of normal rat 40×10
Takamatsu's method

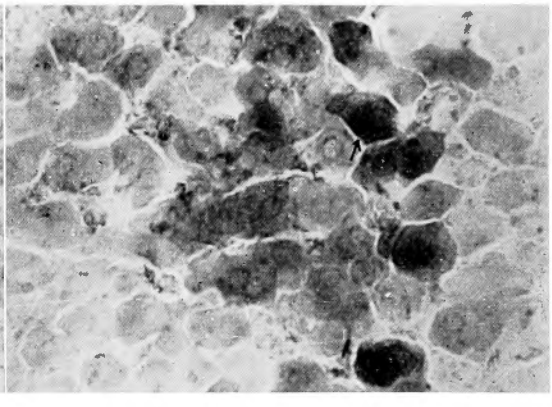

Fig. 23 Liver of normal rat 40×10
Takamatsu's method

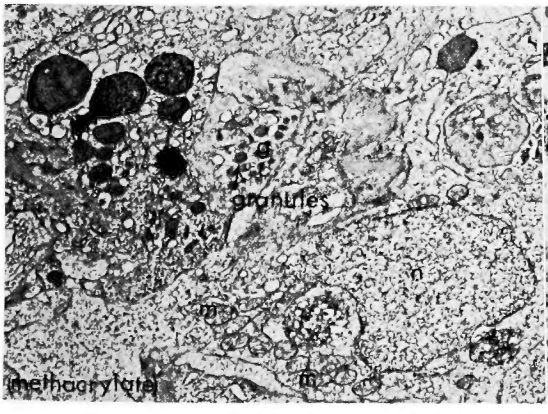

Fig. 24 Electron micrograph of spleen
Rabbit ×14000

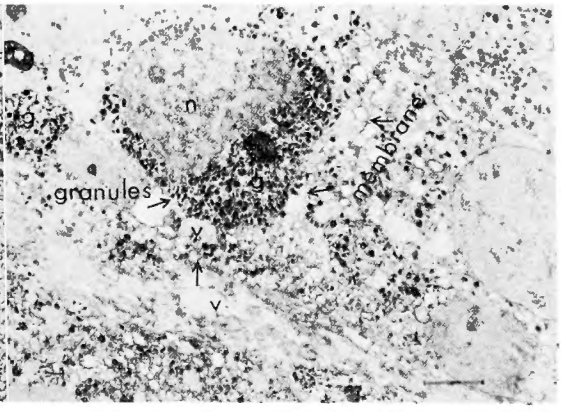

Fig. 25 Electron micrograph of adrenal medulla Rabbit ×14000

Fig. 26 Diffraction pattern of Au

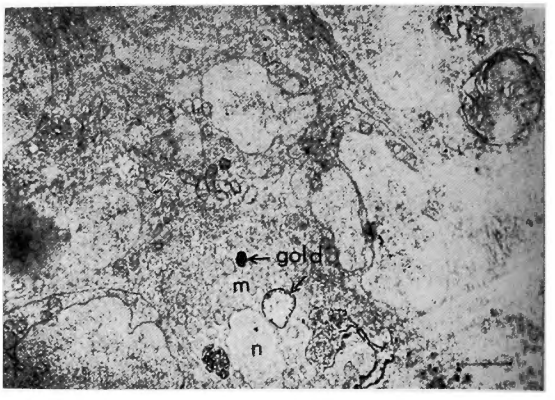

Fig. 27 Electron micrograph of the spleen in the administration study of colloidal gold ×15000

Fig. 28 Diffraction pattern of Au in the spleen (Fig. 27)

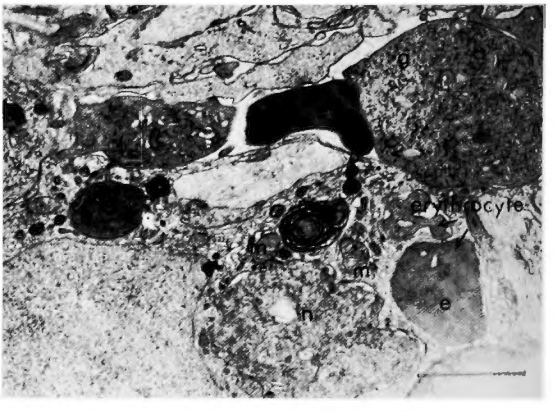

Fig. 29 Electron micrograph of spleen ×30000

Fig. 30 Diffraction pattern of Au in the spleen (Fig. 27)

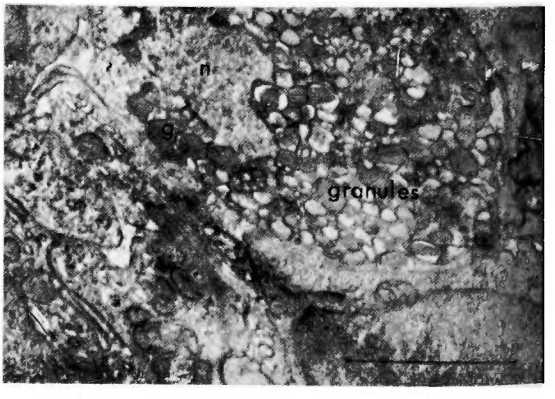

Fig. 31 Electron micrograph of spleen ×45000

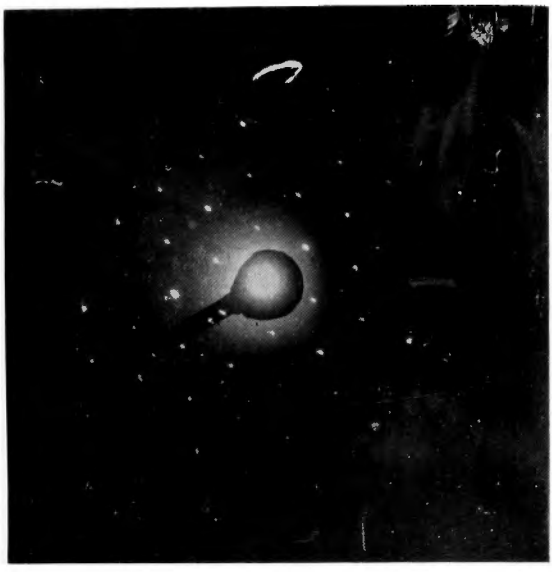

Fig. 32 Diffraction pattern of Au in the liver (Fig. 33)

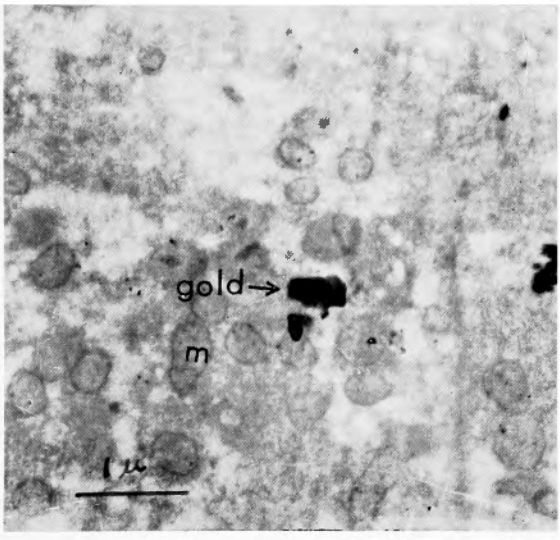

Fig. 33 Electron micrograph of liver ×20000